(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 December 2002 (12.12.2002)

PCT

(10) International Publication Number
WO 02/098914 A2

(51) International Patent Classification[7]: C07K 14/47

(21) International Application Number: PCT/EP02/06161

(22) International Filing Date: 5 June 2002 (05.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
01 112 958.2 7 June 2001 (07.06.2001) EP

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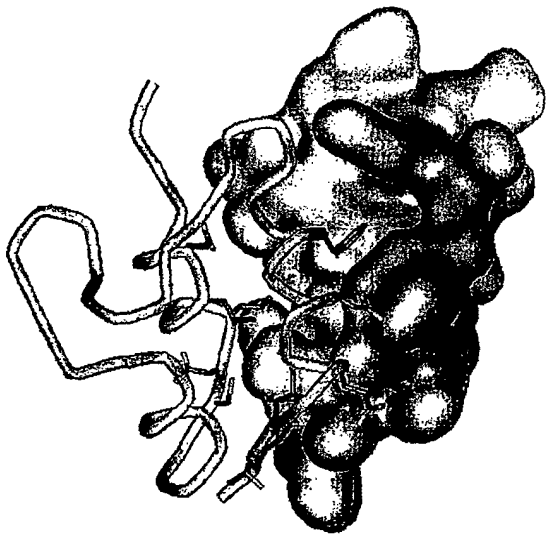
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

[Continued on next page]

(54) Title: MUTANTS OF IGF BINDING PROTEINS AND METHODS OF PRODUCTION OF ANTAGONISTS THEREOF



(57) Abstract: The present invention provides a crystal suitable for X-ray diffraction, comprising a complex of insulin-like growth factor I or II (IGF) and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6; methods for the determination of the atomic coordinates of such a crystal; IGFBP mutants with enhanced binding affinity for IGF-I and/or IGF-II, and methods to identify and optimize small molecules which displace IGFs from their binding proteins.

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European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Mutants of IGF binding proteins and methods of production of antagonists thereof

The present invention relates to a complex of an IGF binding protein fragment (IGFBP) with IGF, its uses and to novel IGFBP mutants with a higher affinity than natural IGFBPs for IGF as well as to methods for the production of antagonists for IGFBPs which hinder or reverse complex formation between IGFBPs and IGF.

Introduction

Insulin-like growth factors I and II (hereafter also referred to as IGFs or IGF) are members of the insulin superfamily of hormones, growth factors and neuropeptides whose biological actions are achieved through binding to cell surface receptors. IGF actions are regulated by IGF binding proteins (IGFBPs) that act as transporters of IGFs, protect them from degradation, limit their binding to receptors, and maintain a "reservoir" of biologically inactive IGF (Martin, J.L., and Baxter, R.C., IGF binding proteins as modulators of IGF actions; in: Rosenfeld, R.G., and Roberts, C.T. (eds.), The IGF system, Molecular Biology, Physiology, and Clinical Applications (1999), Humana Press, Totowa, pp. 227-255; Jones, J.L., and Clemmons, D.R., Endocr. Rev. 12 (1995) 10-21; Khandwala, H.M., et al., Endocr. Rev. 21 (2000) 215-244; Hwa, V., et al., The IGF binding protein superfamily, In: Rosenfeld, R.G., and Roberts, C.T. (eds.), The IGF system, Molecular Biology, Physiology, and Clinical Applications (1999), Humana Press, Totowa, pp. 315-327). The IGF and growth hormone (GH) axis plays a large part in regulating fetal and childhood somatic growth and several decades of basic and clinical research have demonstrated that it also is critical in maintaining neoplastic growth (Khandwala, H.M., et al., Endocr. Rev. 21 (2000) 215-244). High circulating IGF-I concentrations may also be an important determinant of cancer incidence (Hankinson, S.E., et al., Lancet 351 (1998) 1393-1396; Holly, J., Lancet 351 (1998) 1373-1374; Wolk, A., Lancet 356 (2000) 1902-1903). Virtually every level of the IGF system mediating response on the tumor tissues (IGFs, IGFBPs, IGF receptors) can be targeted for therapeutic approaches (Khandwala, H.M., et al., Endocr. Rev. 21 (2000) 215-244; Fanayan, S., et al., J. Biol. Chem. 275 (2000) 39146-39151; Imai, Y., et al., J. Biol. Chem. 275 (2000) 18188-18194). It should also be mentioned here that IGFBP-3 has IGF-independent anti-proliferative and proapoptotic effects (Wetterau, L.A., et al., Mol.

Gen. Metab. 68 (1999) 161-181; Butt, A.J., et al., J. Biol. Chem. 275 (2000) 39174-39181).

IGF-I and IGF-II are 67% identical single polypeptide chains of 70 and 67 amino acids, respectively, sharing with insulin about 40% sequence identity and presumed structural homology. The first 29 residues of IGFs are homologous to the B-chain of insulin (B region, 1-29), followed by 12 residues that are analogous to the C-peptide of proinsulin (C region, 30-41), and a 21-residue region that is homologous to the A-chain of insulin (A region, 42-62). The carboxy-terminal octapeptide (D region, 63-70) has no counterpart in insulins and proinsulins (Murray-Rust, J., et al., BioEssays 14 (1992) 325-331; Baxter, R.C., et al., J. Biol. Chem. 267 (1992) 60-65). The IGFs are the only members of the insulin superfamily in which the C region is not removed proteolytically after translation. The 3D structure of insulin has been studied intensively since the first crystal structure determination in the 1960s (Adams, M.J., et al., Nature 224 (1969) 491-492). There are now structures of insulins in several oligomeric states, for insulins crystallized in different solvent conditions, and for many variants from different species and chemical modifications. This is in stark contrast to IGFs (and proinsulins) for which no high definition structure has been available prior to this report. Instead, the tertiary structure of IGF-I has been modeled after porcine insulin (Blundell, T.L., Proc. Natl. Acad. Sci. USA 75 (1978) 180-184). 2D NMR studies of IGF-I have confirmed that the solution structure is consistent with the model (Cooke, R.M., et al., Biochemistry 30 (1991) 5484-5491; Sato, A., et al., Int. J. Pept. Protein Res. 41 (1993) 433-440). However, NMR studies of IGF-I have yielded structures only of low resolution, probably because IGF-I is soluble at the concentrations required for NMR only at pH values below 3 (Cooke, R.M., et al., Biochemistry 30 (1991) 5484-5491; Sato, A., et al., Int. J. Pept. Protein Res. 41 (1993) 433-440). More recently, better defined structures have been obtained for IGF-II (Terasawa, H., et al., EMBO J. 13 (1994) 5590-5597; Torres, A.M., et al., J. Mol. Biol. 248 (1995) 385-401) and for a Glu-3 to Arg variant of IGF-I (long-[Arg3]IGF-I) that additionally possesses a 13-amino acid extension at the N-terminus (Laajoki, L.G., et al., J. Biol. Chem. 275 (2000) 10009-10015).

IGFBPs (insulin-like growth factor binding proteins -1 to -6) are proteins of 216 to 289 residues, with mature IGFBP-5 consisting of 252 residues (Wetterau, L.A., et al., Mol. Gen. Metab. 68 (1999) 161-181). All IGFBPs share a common domain

organization. The highest conservation is found in the N- (residues 1 to ca. 100) and C- (from residue 170) terminal cysteine rich regions. Twelve conserved cysteines are found in the N-terminal domain and six in the C-terminal domain. The central, weakly conserved part (L-domain) contains most of the cleavage sites for specific proteases (Chernausek, S.D., et al., J. Biol. Chem. 270 (1995) 11377-11382). Several different fragments of IGFBPs have been described and biochemically characterized so far (Mazerbourg, S., et al., Endocrinology 140 (1999) 4175-4184). Mutagenesis studies suggest that the high affinity IGF binding site is located in the N-terminal domain (Wetterau, L.A., et al., Mol. Gen. Metab. 68 (1999) 161-181; Chernausek, S.D., et al., J. Biol. Chem. 270 (1995) 11377-11382) and that at least IGFBP-3 and IGFBP-2 contain two binding determinants, one in the N- and one at the C-terminal domains (Wetterau, L.A., et al., Mol. Gen. Metab. 68 (1999) 161-181). Recently, a group of IGFBP-related proteins (IGFBP-rPs) which bind IGFs with lower affinity than IGFBPs have been described (Hwa, V., et al., The IGF binding protein superfamily, In: Rosenfeld, R.G., and Roberts, C.T. (eds.), The IGF system, Molecular Biology, Physiology, and Clinical Applications (1999), Humana Press, Totowa, pp. 315-327). IGFBPs and IGFBP-rPs share the highly conserved and cysteine-rich N-terminal domain which appears to be crucial for several biological actions, including their binding to IGFs and high affinity binding to insulin (Hwa et al., 1999). N-terminal fragments of IGFBP-3, generated for example by plasma digestion, also bind insulin and physiologically are thus likely relevant for insulin action. Beyond the N-terminal domain, there is a lack of sequence similarity between the IGFBPs and IGFBP-rPs.

The sequences of human IGFBP-1 to -6 are described in detail in the SwissProt Database (http://www.expasy.ch) and identified by the following Accession Nos.:

Name	Accession No.
IGFBP-1	P 08833
IGFBP-2	P 18065
IGFBP-3	P 17936
IGFBP-4	P 22692
IGFBP-5	P 24593
IGFBP-6	P 24592

The amino acid positions described in the following refer to the sequence of the mature forms the human IGF binding proteins (sequence after removal of the signaling peptide starts with amino acid in position 1, see also Tables 1 to 6).

The association of insulin-like growth factors with neoplasia indicates that inhibition of the IGF signaling pathway in tumors might be a successful strategy in cancer therapy. Such modulation might be accomplished, for example, through exogenous administration of recombinant inhibitory IGFBPs and effective fragments thereof. Additionally, tumor cell IGFBP production, inhibition or degradation may be controlled by agents such as tamoxifen and ICI 182,780 that modify tumor IGFBP production (Khandwala, H.M., et al., Endocr. Rev. 21 (2000) 215-244). The consequent alteration in IGFBP-3 levels appears in certain instances to inhibit IGF-I-stimulated cell proliferation (Khwandala et al., 2000). There is also recent evidence that IGFBP-3 may be a p53-independent effector of apoptosis in breast cancer cells via its modulation of the Bax:Bcl-2 protein ratio (Butt, A.J., et al., J. Biol. Chem. 275 (2000) 39174-39181; Wetterau, L.A., et al., Mol. Gen. Metab. 68 (1999) 161-181).

IGFBPs show a significant inhibition of tumor cell proliferation in vitro but only very high doses result in inhibition of tumor growth in vivo (van den Berg, C.L., et al., Eur. J. Cancer 33 (1997) 1108-1113). Van den Berg therefore covalently coupled IGFBP-1 to polyethylene glycol, which leads to a prolonged serum half-life. However, the inhibitory effects of the pegylated IGFBP-1 is still not sufficient for therapeutic intervention in humans because only partial response is observed even if pegylated IGFBP-1 is given in doses of 1 mg/dose daily in mice. This corresponds to a dose of 50 mg/kg x day which can not be administered to humans by established procedures and can not be produced economically.

IGF releasing peptides are described by Loddick, S.A., et al., Proc. Natl. Acad. Sci. USA 95 (1998) 1894-1898 and Lowman, H.B., et al., Biochemistry 37 (1998) 8870-8878. The described molecules which are able to displace IGFs from their binding proteins are either mutants of IGF-I which bind to IGFBPs but are not able to stimulate the IGF-IR or a 14 amino acid peptide with similar properties derived from a phage-display library. The biological activities of the peptides were shown by administration either by injection into the lateral ventricle of the brain or infused by a minipump.

Mutagenesis studies for IGFs indicated that IGF amino acid residues Glu 3, Thr 4, Gln 15 and Phe 16 of IGF-I and Glu 6, Phe 48, Arg 49 and Ser 50 in IGF-II are important for binding to IGFBPs (Baxter, R.C., et al., J. Biol. Chem. 267 (1992) 60-65; Bach, L.A., et al., J. Biol. Chem. 268 (1993) 9246-9254; Luethi, C., et al., Eur. J. Biochem. 205 (1992) 483-490; Jansson, M., et al., Biochemistry 36 (1997) 4108-4117). Baxter et al. (1992) suggested that the IGF-I amino acid residues Glu 3, Thr 4, Gln 15 and Phe 16 are crucial for interaction with IGFBP-3, whereas residues Phe 49, Arg 50 and Ser 51 are of secondary importance. It also was suggested that Phe 26 of IGF-II plays a role in changing the local structures of IGFs but does not directly bind to IGFBPs (Terasawa, H., et al., EMBO J. 13 (1994) 5590-5597).

Kalus, W., et al., in EMBO J. 17 (1998) 6558-6572, describe proteolytic studies of human IGFBP-5 and the cloning and expressing of the domain of IGFBP-5 between residues 40-92 (mini-IGFBP-5); this domain binds IGF-I and IGF-II with K_D values of 37 nM and 6 nM, respectively, as well as the determination of the solution structure of uncomplexed mini-IGFBP-5 by NMR. Kalus et al. identified some IGF binding sites which are residues Val49, Tyr50, Pro62 and Lys68 to Leu75 of IGFBP-5.

Imai, Y., et al., in J. Biol. Chem. 275 (2000) 18188-18194, describe an IGFBP-3 variant and an IGFBP-5 variant, each with a five-fold substitution pattern at amino acid positions hypothesized by Kalus et al. as IGF binding sites. Imai et al. found that a substantial alteration of the amino acid residues simultaneously at positions 68, 69, 70, 73 and 74 results in a 1000-fold or larger reduction in the affinity for IGF-I in relation to the affinity of wild-type IGFBP-5.

Conover, C.A., et al., in J. Biol. Chem. 270 (1995) 4395-4400, describe protease-resistant mutants of IGFBP-4. All four IGFBP-4 mutants around the putative cleavage site at Met135-Lys136 and the wild-type protein bind IGFs with equivalent affinities.

Byun, D., et al., in J. Endocrinology 169 (2001) 135-143, postulate several regions involved in IGF binding by IGFBP-4. Deletion of segments Leu72-Ser 91 or Leu72-His74 results in loss of IGF binding. Also mutation of certain cysteine residues significantly reduces the binding of IGFs.

Thus, these described mutant forms of insulin-like growth factor binding proteins have reduced or equivalent affinities for IGF-I and/or IGF-II. Mutants of IGFBPs with a significantly higher affinity and a therefore improved effectiveness have not been known heretofore and there exists a need for such molecules as well as for methods for identifying IGFBP antagonists.

Summary of the Invention

The invention provides a crystal suitable for X-ray diffraction, comprising a complex of insulin-like growth factor I or II and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9^{th} to 12^{th} cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7^{th} to 10^{th} cysteine of IGFBP-6 (such polypeptides and fragments are hereinafter also referred to as "mini-IGFBPs).

Such a crystal is suitable for determining the atomic coordinates of the binding sites of IGF-I, IGF-II, and IGFBPs, and therefore allows the optimization of these molecules to identify and improve stabilizing interactions between IGF-I or IGF-II and IGFBPs. Preferably, the crystal effectively diffracts X-ray for the determination of the atomic coordinates of said complex to a resolution of 1.5 to 3.5 Å. The crystal is arranged in the cubic space group $P2_13$ having unit cell dimensions of 74.385 Å x 74.385 Å x 74.385 Å.

The invention further provides a method for producing a crystal suitable for X-ray diffraction, comprising

(a) contacting IGF-I or IGF-II with a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40–92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9^{th} to 12^{th} cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7^{th} to 10^{th} cysteine of IGFBP-6, to form a complex which exhibits restricted conformation mobility, and

(b) obtaining a crystal from the complex so formed suitable for X-ray diffraction.

Using this crystal, the atomic coordinates of the complex can be determined.

The invention further comprises a method for identifying a mutant of IGFBP or a mutant of a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, and having enhanced binding affinity for IGF-I and/or IGF-II comprising

(a) constructing a three-dimensional structure of the complex of IGF-I or IGF-II and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40–92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, based on the atomic coordinates of a crystal consisting of IGF-I or IGF-II and said polypeptide;

(b) employing said three-dimensional structure and modeling methods to identify said mutant in which an amino acid residue within a distance of 5 Å to a hydrophobic amino acid residue of IGF-I or IGF-II is modified in that the hydrophobic interaction between IGF-I or IGF-II and said mutant of IGFBP is enhanced;

(c) producing said mutant;

(d) assaying said mutant to determine said enhanced binding affinity for IGF.

The invention further comprises a method for identifying a mutant of IGFBP-5 with enhanced binding affinity for IGF-I, said method comprising

(a) constructing a three-dimensional structure of the complex of IGF-1 and IGFBP-5 defined by the atomic coordinates shown in Figs. 5 and 6;

(b) employing said three-dimensional structure and modeling methods to identify an amino acid residue in IGFBP-5 within a distance of 5 Å or shorter to an amino acid residue of IGF-I, wherein said residue of IGFBP-5 can be modified hydrophobically in that the hydrophobic interaction between IGF and IGFBP-5 is enhanced;

(c) producing said mutant;

(d) assaying said mutant to determine said enhanced binding affinity for IGF.

The amino acid residue(s) in which IGFBP(s) is/are modified is/are preferably selected from the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 49-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6.

Especially preferred IGFBP mutants are modified at amino acid residues 49, 70 and/or 73 corresponding to IGFBP-5 sequence alignment and according to Table 7.

The invention therefore provides mutant IGFBPs ("IGFBPs" as used herein means IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and/or IGFBP-6) with enhanced affinity (preferably about 3-fold to 10-fold increased affinity to the corresponding wild-type IGFBP) for IGF ("IGF" as used herein means IGF-I and/or IGF-II), improved inhibitory potency for the activity of IGF in vitro and in vivo and therefore improved therapeutic effectiveness.

The invention further provides a method for identifying a compound capable of binding to IGFBP, comprising

(a) constructing a three-dimensional structure of a complex of IGF-I or IGF-II and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, amino acids 55-107 of IGFBP-2, amino acids 47-99 of IGFBP-3, amino acids 39-91 of IGFBP-4, amino acids 40-92 of IGFBP-5, amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9^{th} to 12^{th} cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7^{th} to 10^{th} cysteine of IGFBP-6, based on the atomic coordinates of a crystal consisting of IGF – I and said IGFBP;

(b) employing said three-dimensional structure and modeling methods to identify a compound forming a complex with said IGFBP by hydrophobic binding with amino acids 49, 50, 70, 71 and 74 in the case of IGFBP-5 and in the case of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-6 with corresponding amino acids according to Table 7;

(c) producing said compound;

(d) determining the binding between the compound and IGFBP.

The invention further provides a method of inhibiting the binding of IGF to the IGFBP in a subject, preferably a human subject, comprising administering an effective amount of an above-described mutant of IGFBP to the subject.

Detailed Description of the Invention

The present invention provides methods for co-crystallizing IGF-I or IGF-II with a truncated N-terminal fragment of IGFBP, preferably of IGFBP-5 (mini-IGF), where the crystals diffract X-rays with sufficiently high resolution to allow determination of the three-dimensional structure of said complex, including atomic coordinates. The three-dimensional structure (e.g. as provided on computer-readable media) is useful for rational drug design of IGFBP mutants with modified affinity for IGF-I or IGF-II, preferably with an improved affinity. There is specifically provided a method for co-crystallizing IGF-I with a polypeptide consisting of an isolated folded domain of IGFBPs (mini-IGFBPs), which is formed by the amino acids between the 9^{th} and the 12^{th} cysteine of IGFBP-1 to IGFBP-5 or the 7^{th} and 10^{th} cysteine of IGFBP-6 and additionally including up to 7 amino acids N-terminal of this fragment and up to 5-20 amino acids C-terminal to this fragment. The amino acids 39-91of BP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or fragments thereof are especially suitable to form a complex with IGF-I or IGF-II which exhibits restricted conformational mobility and high suitability for X-ray diffraction.

Such a complex co-crystallizes in a manner sufficient for the determination of atomic coordinates by X-ray diffraction. The crystal effectively diffracts X-ray for the determination of the atomic coordinates of the complex to a resolution of 1.5 or at least better (less) than 3.5 Å. Said IGFBP fragments are able to form a compact and globular structure whose scaffold is secured by an inside packing of two cysteine bridges and stabilized further by a three-stranded ß-sheet. The folded fragments are still able to bind IGF-I and IGF-II with high affinities. Other forms of the IGFBPs such as full-length IGFBPs, the isolated C-terminal domain of IGFBPs or fragments without N-terminal truncation do not co-crystallize with IGF in a suitable manner for X-ray-based determination of the structure at high resolution.

Knowledge of the crystal structure enables the production of specific IGFBP mutants which develop improved interaction with, thereby exhibiting enhanced affinity for, IGF and, as a consequence, have improved therapeutic efficacy as IGF antagonists. Such IGFBP mutants with increased affinity for IGF are capable of preventing the formation of the complex between naturally occurring IGF and IGF-I receptor (IGF-IR) in vitro and in vivo and, thereby, of effecting an decrease in the concentration of biologically active, free IGF. Such rational designed IGF antagonists are therefore capable of inhibiting tumor growth and inducing apoptosis in tumor cells more efficient than natural IGFBPs. As a result, lower doses of the optimal designed IGFBP mutants with enhanced affinity are needed for achieving an effect comparable to that of naturally occurring IGFBPs.

A further embodiment of the invention is the identification and optimization of non-proteinaceous compounds which bind to the IGF binding site of IGFBPs and prevent the formation of an inhibitory complex between IGFs and IGFBPs and therefore activates the anabolic action of IGF. Such "IGF-releasing compounds" can be identified according to the invention on the basis of the crystal data, using protein-ligand docking programs such as FlexX (Kramer, B., et al., Proteins: Structure, Functions and Genetics 37 (1999) 228-241).

The X-ray diffraction patterns of the invention have a sufficiently high resolution to be useful for three-dimensional modeling of an IGF releasing compound. Preferably, the resolution is in the range of 1.5 to 3.5 Å, preferably 1.5 to 3.0 Å. Three-dimensional modeling is performed using the diffraction coordinates from these X-ray diffraction patterns. The coordinates are entered into one or more computer programs for molecular modeling as known in the art. Such molecular modeling can utilize known X-ray diffraction molecular modeling algorithms or molecular modeling software to generate atomic coordinates corresponding to the three-dimensional structure of at least one IGF releasing compound.

Such a compound shows affinity for IGFBP based on stereochemical complementary relative to naturally occurring IGFs. Such stereochemical complementary according to the present invention is characterized by a molecule that matches intra-site surface residues that form the contours of IGFBPs as enumerated by the coordinates set out in Figs. 5 and 6. The residues that define the contours are depicted in Figs. 5 and 6. Matching according to the invention means

that the identified atoms or atom groups interact with the IGFBP surface residues, for example via hydrogen bonding or by enthalpy-reduced van der Waals interactions which prevent or reduce the interaction between IGFBP and IGFs and thereby promote the release of the biologically active compound from the binding site. In general, the design of a molecule possessing stereochemical complementary to the contours of IGFBPs can be accomplished by means of techniques that optimize either chemically or geometrically the fit between a molecule and a target receptor. Known techniques of this sort are reviewed by Sheridan, R.P., and Venktaraghavan, R., Acc. Chem. Res. 20 (1987) 322; Goodford, P.J., J. Med. Chem. 27 (1984) 557; Verlinde, C., and Hol, W., Structure 2 (1994) 577; and Blundell, T.L. et al., Nature 326 (1987) 347. The design of optimized IGFBP ligands based on the invention is preferably done by the use of software such as GRID (Goodford, P.J., J. Med. Chem. 28 (1985) 849-857), a program that determines probable interaction sites between probes with various functional group characteristics and the protein surface - is used to analyze the surface sites to determine structures of similar inhibiting proteins or compounds.

The program DOCK (Kuntz, I.D., et al., J. Mol. Biol. 161 (1982) 269-288) can also be used to analyze an active site or ligand binding site and suggest ligands with complementary steric properties. Several methodologies for searching three-dimensional databases to test pharmacophore hypotheses and select compounds for screening are available. These include the program CAVEAT (Bacon et al., J. Mol. Biol. 225 (1992) 849-858) which uses databases of cyclic compounds which can act as spacers to connect any number of chemical fragments already positioned in the active site. The program LUDI (Bohm, H.J., et al., J. Comput. Aided Mol. Des. 6 (1992) 61-78 and 593-606) defines interaction sites into which both hydrogen bonding and hydrophobic fragments fit.

Programs suitable for searching three-dimensional databases to identify also non-proteinaceous molecules bearing a desired pharmacophore include: MACCS-3D and ISIS/3D (Molecular Design Ltd., San Leandro, CA), ChemDBS-3D (Chemical Design Ltd., Oxford, U.K.), and Sybyl/3DB Unity (Tripos Associates, St. Louis, MO).

Programs suitable for pharmacophore selection and design include: DISCO (Abbott Laboratories, Abbott Park, IL), Catalyst (Bio-CAD Corp., Mountain View, CA), and ChemDBS-3D (Chemical Design Ltd., Oxford, U.K.).

Databases of chemical structures are available from a number of sources including Cambridge Crystallographic Data Centre (Cambridge, U.K.) and Chemical Abstracts Service (Columbus, OH).

De novo design programs include Ludi (Biosyrn Technologies Inc., San Diego, CA), Sybyl (Tripos Associates) and Aladdin (Daylight Chemical Information Systems, Irvine, CA).

Those skilled in the art will recognize that the design of such compounds may require slight structural alteration or adjustment of a chemical structure designed or identified using the methods of the invention.

Non-proteinaceous compounds and IGFBP mutants with increased binding affinity for IGF can be identified by incubating said compounds or mutants with an IGF-I/IGFBP-5 complex and measuring the binding of released IGF-I to IGF-I receptor expressing cells. Due to the binding of IGF-I to its cell-bound receptor, the receptor is activated and autophosphorylated. Alternatively, radiolabeled IGF-I can be used and its binding to its receptor after release from the complex can be determined.

Formation of the IGF-I mini-IGFBP-5 complex buries a binding surface totalling about 550 $Å^2$. Of the eleven IGFBP-5 amino acid residues within 5 Å of IGF, six are hydrophobic, three of which are surface-exposed leucines and valines and are of primary importance for hydrophobic interaction to IGFs (Figures 1 to 4). On the IGF side, four of the eleven amino acid residues within 5 Å of mini-IBFBP-5 are hydrophobic (Figures 1 to 4).

The IGFBPs bind to IGF-I and IGF-II by presenting a binding surface complementary to that of IGF. The IGF binding surface consists of a relatively flat hydrophobic surface, a small hydrophobic depression, two hydrophobic protruberances, and surrounding polar residues. Identification of the IGF binding surface itself (Figure 3) enables the design of binding partners in general, and

optimization of known binding partners in particular. General binding partners will have at least two of the following features 1 to 4:

1. Non-polar atoms lying approximately in a plane defined by atoms Leu74 CD1 and CD2, Val49 CG1 and CG2, Leu70 CB, and Tyr 50 CB, within a perimeter defined by IGF residues Glu9, Glu3, Leu54, Phe 16 and by BP5 atom Tyr 50 OH and depicted in Figure 3 such that they present an approximately planar and hydrophobic molecular surface of at least 20 square Angstroms.

2. A non-polar atom or atoms near the positions of Leu 70 CG, CD1 relative to IGF, filling the depression of IGF as seen in the complex structure.

3. Hydrophobic and/or aromatic interactions with the side chains of Phe16, Val17, and/or Leu54 of IGF as defined by a net buried surface area in the complex of at least 20 square Angstroms.

4. Polar (hydrogen bonding and/or charge complementary) interactions, either directly or via bridging solvent molecules, with one or more of the following IGF atoms: Asp12 OD1,2; Glu9 OE1,2; Glu3 OE1,2; Glu58 OE1,2; Thr4 O,OG1; Cys52O; Ser51 OG; Asp53OD1,2; Arg55NH1,2,NE; Arg21NH1,2,NE; Val17O; Cys18O; Asp20OD1,2,N; Gln15O,OD1,ND2.

Abbreviations: Letters corresponding to standard amino acid atom naming (according to the International Union of Physicists and Chemists—IUPAC—naming).

CG:	Carbon Cγ
CB:	Carbon Cβ
OE:	Oxygen Oε
OH:	Oxygen Oη
OD:	Oxygen Oδ
O:	Backbone Oxygen
NH:	Nitrogen Nη
NE:	Nitrogen Nε
N:	Backbone Nitrogen
ND:	Nitrogen Nδ

The principal IGF/IGFBP interaction, shown in the example of IGF-I mini-IGFBP-5 interaction, is a hydrophobic sandwich that consists of interlaced protruding side chains of IGF-I and solvent exposed hydrophobic side chains of the mini-IGFBP-5 (Figures 1 to 4). The side-chains of IGF-I Phe 16, Leu 54 and also Glu 3, are inserted deep into a cleft on the mini-IGFBP-5 (Figures 1 to 4). This cleft is formed by side chains of Arg 53, Arg 59 on the solvent exposed side of the molecule and by Val 49, Leu 70, Leu 74 on the opposite inner side, with a base formed by residues Cys 60 and Leu 61. Phe 16 makes direct contacts with the backbone and side chain of Val 49, and with Cys 60 of mini-IGFBP-5. The hydrophobic cluster is closed on the solvent side by side chains of Glu 3 and Glu 9 of IGF-I and His 71 and Tyr 50 of mini-IGFBP-5. These residues form a network of hydrogen bonds; in addition Arg 59 of mini-IGFBP-5 makes hydrogen bonds with Glu 58 (Figures 2 to 4).

Arg 53 and Arg 59 of mini-IGFBP-5 isolate the hydrophobic sandwich from the solvent close to the C-terminus. In the full length IGFBP-5, the segment corresponding to the C-terminus of mini-IGFBP-5 is followed by nine hydrophilic residues and then by at least 30 residues of mixed types. Thus, the conformations seen in the structure of the complex near the C-terminus of mini-IGFBP-5 are likely to be preserved in the complex of IGF-I with the full length-IGFBP-5. The mini-IGFBP-5 domain begins preferably at residue 40 of full length IGFBP-5.

The hydrophobic residues Val 49, Leu 70 and Leu 73 of IGFBP-5 are crucial for binding to IGFs. Since these residues are highly conserved among all IGFBPs, these hydrophobic interactions dominate the IGF binding properties of all IGFBPs.

The increased inhibitory potency of the mutant IGFBPs and fragments thereof results in the inhibition of the binding to and autophosphorylation of the IGF-IR (as described by Kalus, W., et al., in EMBO J. 17 (1998) 6558-6572) at significantly lower concentrations than observed for the wildtype proteins and the corresponding fragments. The higher potency of the mutant IGFBPs furthermore can be shown by the inhibition of the growth of tumor cells in vitro and in vivo. The growth of several tumor cell lines is known to be significantly reduced by IGFBPs. IGFBP-1 for example inhibits the growth of MCF-7 and MDA-MB-435A cells in vitro and the growth of tumors formed MDA-MB-231 cells in vivo in mice

(van den Berg, C.L., et al., Eur. J. Cancer 33 (1997) 1108-1113). IGFBP mutants with increased affinity inhibit the growth of these tumor cells at lower concentrations than the wild type proteins.

The following mutations of IGFBPs are preferred for enhancing binding affinity to IGF (numbering according to IGF-BP5 as aligned in Fig. 1) (standard one-letter abbreviation for amino acids used):

Table 1:

IGFBP-1

Amino acid No.	Original amino acid	Preferred mutations[1)]
48	V	L,I,M,F,Y,W
49	A	Y,R,K
52	R	W,Y,M,F,H
60	R	Y,W,F
69	L	Y,W,M,I,F
72	L	I,Y,W,M,F
73	T	V,L,Y,W,M,I,F
74	R	H,D
82	E	R,K,H,N,Q,S,T,A,G

Table 2:

IGFBP-2

Amino acid No.	Original amino acid	Preferred mutations[1)]
64	V	L,I,M,F,Y,W
65	Y	R,K
68	R	W,Y,M,F,H
76	Y	W,F
85	L	Y,W,M,I,F
86	Q	T,S,R,K,N,H,Y,C
88	L	I,Y,W,M,F
89	V	L,I,Y,W,M,F
90	M	H,D

Table 3:

IGFBP-3

Amino acid No.	Original amino acid	Preferred mutations[1)]
56	I	L,V,M,F,Y,W
57	Y	R,K
60	R	W,Y,M,F,H
68	Q	L,Y,W,F
75	R	Q
77	L	Y,W,M,I,F
78	Q	T,S,R,K,N,H,Y,C
80	L	I,Y,W,M,F
81	L	Y,W,M,I,F

Table 4:

IGFBP-4

Amino acid No.	Original amino acid	Preferred mutations[1)]
48	V	L,I,M,F,Y,W
49	Y	R,K
52	R	W,Y,M,F,H
60	Y	W,F
67	K	Q
69	L	Y,W,M,I,F
72	L	I,Y,W,M,F
73	M	Y,W,I,F
74	H	D

Table 5:

IGFBP-5

Amino acid No.	Original amino acid	Preferred mutations[1)]
49	V	L,I,M,F,Y,W
50	Y	R,K
53	R	W,Y,M,F,H
61	L	Y,W,F
68	K	Q
70	L	Y,W,M,I,F
73	L	I,Y,W,M,F
74	L	Y,W,M,I,F
75	H	D
83	E	R,K,H,N,Q,S,T,A,G

Table 6:

IGFBP-6

Amino acid No.	Original amino acid	Preferred mutations[1)]
49	V	L,**I**,M,F,Y,W
50	Y	R,**K**
53	N	R,W,**Y**,M,F,H
61	H	L,Y,W,F
68	A	K,Q
70	L	Y,W,M,I,F
71	R	T,S,**H**,K,N,Q,Y,C
73	L	I,Y,**W**,M,I,F
74	L	Y,W,M,I,F
75	L	H,D

1) Amino acids are given in the standard one-letter amino acid code and are to be understood as alternative amino acid exchanges (or). For instance, the last line of Table 6 means that amino acid residue 75 of IGFBP-6, which is leucine (L), can preferably be modified to be either histidine (H) or aspartic acid (D). Table 6 is additionally to be interpreted such that amino acids 49, 50, 53, 61, 68, 70, 73, 74 and/or 75 can be exchanged in order to improve affinity. Especially preferred are IGFBP mutants with single point mutations. Most preferred are IGFBP mutants having a single point mutation from the bold face residues. This applies correspondingly to the other tables.

Table 7:

Sequence alignment

showing corresponding amino acids of IGFBP-1 to -6

Amino Acid No.					
IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
48	64	56	48	49	49
49	65	57	49	50	50
52	68	60	52	53	53
60	76	68	60	61	61
67	83	75	67	68	68
69	85	77	69	70	70
70	86	78	70	71	71
72	88	80	72	73	73
73	89	81	73	74	74
74	90	82	74	75	75

The presented structure enables in silico screens for small IGFBP ligand inhibitors with the potential to release "free" bioactive IGF. Displacement of IGF from their binding proteins are therapeutically useful in treating a variety of potential indications, including short stature, renal failure, type I and type II diabetis, stroke and other neuro-degenerative diseases.

The compounds and IGFBP mutants of the present invention can be formulated according to methods for the preparation of compositions, preferably pharmaceutical compositions, which methods are known to the person skilled in the art. Preferably, such a compound and IGFBP mutant is combined in a mixture with a pharmaceutically acceptable carrier. Such acceptable carriers are described in, for example, Remington's Pharmaceutical Sciences, 18th ed., 1990, Mack Publishing Company, edited by Oslo et al. (e.g. pp. 1435-1712). Typical compositions contain an effective amount of a non-proteinaceous compound or IGFBP mutant according to the invention, for example from about 1 to 10 mg/ml, together with a suitable amount of a carrier. The compounds and IGFBP mutants may be administered preferably parenterally.

The invention further provides pharmaceutical compositions containing a non-proteinaceous compound or IGFBP mutant according to the invention. Such pharmaceutical compositions contain an effective amount of a compound and IGFBP mutant of the invention, together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer contents (e.g., acetate, phosphate, phosphate-buffered saline), pH and ionic strength, additives such as detergents and solubilizing agents (e.g., Tween®80, polysorbate, Pluronic®F68), antioxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (Timersol®, benzyl alcohol) and bulking substances (e.g., saccharose, mannitol).

Compositions and pharmaceutical compositions according to the invention are manufactured in that the substances in pure lyophilized form are dissolved at a concentration up to from 1 to 20 mg/l in PBS or physiological sodium chloride solution at a neutral pH value. For better solubility it is preferred to add a detergent.

Typically, in a standard cancer treatment regimen, patients are treated with dosages in the range of between 0.5 to 10 mg substance/kg weight per day.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1A: Sequence alignment of IGF-I and IGF-II. Bold underlined residues of IGF-I make contacts with mini-IGFPB5. Residues responsible for binding to the IGF-I receptor (IGF-IR) are marked with an asterisk above the sequence.

Figure 1B: Multiple sequence alignment of the N-terminal domains of human IGF-BPs 1-6. The mini-BP construct, numbered according to BP5 numbering, is marked above the aligned residues with „m“, including GS which indicate additional

residues from the cloning vector. (After position 81, mini-BP5 was disordered in the X-ray structure; this is indicated with italics.) BP5 residues that interact with IGF-I are shown underlined and in bold face. The degree of conservation of the residues is marked under the alignment with * for strict conservation, : for strict conservation of residue type, and . for relatively high conservation. The consensus sequence uses the following code to depict level of strict conservation: o alcohol, l aliphatic, a aromatic, c charged, h hydrophobic, - negative, p polar, + positive, s small, u tiny, t turnlike).

Figure 2: The overall structure of the IGF-I (tube model) mini-IGFBP5 (molecular surface) complex. Side chains plotted show the IGF residues in contact with BP5. Particularly important is Phe16, seen filling a hydrophobic depression on the BP5 surface.

Figure 3: Similar to Figure 2, whereby the IGF is depicted with its molecular surface and BP5 is depicted as a tube model. Side chains of BP5 responsible for binding to IGF are also depicted. The surface of IGF Phe16 is prominent, as is the relatively flat hydrophobic IGF surface contributing to the interface.

Figures 4A and 4B: Summary of IGF-BP5 and IGF-I contacts. Interactions contributing to the binding affinity consist of hydrophobic interactions (a) (involving especially residues Leucines 70, 73, and 74 of BP5 and Phe16 of IGF-I) and also polar interactions (b). Enhancement of BP-IGF binding relies especially on the enhancement of hydrophobic interactions, either by increasing the intermolecular contact surface with these or with additional residues, or by the introduction of further polar contacts.

(A) Packing contacts between IGFBP-5 and IGF-I. Contacts are denoted according to nearest distances, whereby the closest contacts include polar interactions.

(B) Polar contacts between IGFBP-5 and IGF-I. Abbreviations denote hydrogen bonds (HB), CH-O hydrogen bonds (CHB), salt

bridge (SB), and side chain (SC) or main chain (MC) interactions.

Figure 5: Atomic coordinates of IGF-I in the complex with mini-IGFBP-5.

Figure 6: Atomic coordinates of mini-IGFBP-5 in the complex with IGF-I.

Figure 7: Binding of radioactive J-125 IGF-I to NIH 3T3 cells expressing the IGF-IR in the absence and in the presence of IGFBP-5 and compounds potentially interfering with complex formation between IGF-I and IGFBP-5

Figure 8: IGF-I induced autophosphorylation of the IGF-IR expressed by NIH 3T3 cells in the absence and in the presence of IGFBP-5 and compounds potentially interfering with complex formation between IGF-I and IGFBP-5

Sequence Listing

SEQ ID NO:1 Primer FBP5LY.

SEQ ID NO:2 Primer RBP5LY.

SEQ ID NO:3 Primer FBP5LM.

SEQ ID NO:4 Primer RBP5LM.

SEQ ID NO:5 Primer IBP4NdeI.

SEQ ID NO:6 Primer IBP4BamHI.

SEQ ID NO:7 Peptide GSALA.

SEQ ID NO:8 Peptide GSHMDEAIH.

Example 1

Crystallization, data collection and derivatization

Mini-IGFBP-5 was produced as described by Kalus, W., et al., in EMBO J. 17 (1998) 6558-6572 and in Example 6, and IGF-I was obtained from OvoPepi, Australia. Crystallization was successful with 10% Jeffamine M-600, 0.1 M sodium citrate, 0.01 M ferric chloride, pH 5.6. Within 11 days, crystals appeared at 4 °C, growing to a final size of about 0.3 x 0.2 x 0.2 mm3. They belong to the cubic space group P213 and have unit cell dimensions a, b, c = 74.385 Å, with one complex molecule per asymmetric unit. Soaking in precipitation buffer with heavy atom compounds yielded a derivative K2PtCl4 (2.7 mM, 3 d) which was interpretable. All diffraction data were collected using a 300 mm MAR Research (Hamburg, Germany) image plate detector mounted on a Rigaku (Tokyo, Japan) RU300 rotating anode X-ray generator with graphite monochromatized CuKα radiation. All image plate data were processed with MOSFLM (Leslie, A.G.W., Molecular Data in Processing, in: Moras, D., Podjarny, A.D., and Thierry, J.C. (eds.), Crystallographic Computing 5 (1991), Oxford University Press, Oxford, UK, pp. 50-61) and the CCP4 program suite (Collaborative Computational Project, Number 4 1994).

Example 2

Phase calculation, model building and refinement

The structure of the IGF/mini-IGFBP-5 complex was solved by the single isomorphous replacement (s.i.r.) method using one heavy atom derivative described above. Derivative data was analyzed with the native data set, first using isomorphous difference Patterson maps and employing the Patterson vector superposition methods implemented in SHELX-97 (Sheldrick, G., Tutorial on automated Patterson interpretation to find heavy atoms, in: Moras, D., Podjarny, A.D., and Thierry, J.C. (eds.), Crystallographic Computing 5 (1991), Oxford University Press, Oxford, UK, pp. 145-157). The 2 heavy sites locations were confirmed by difference Fourier methods with appropriate initial single site s.i.r. phases using CCP4 programs. The refinement of heavy atom parameters and calculation of s.i.r. phases were done with SHARP (de la Fortelle, E., and de Bricogne, G., Methods Enzymol. 276 (1997) 472-494). The final parameters are given in Table 8. The initial s.i.r. phases were improved with SOLOMON

(Abrahams, J.P., and Leslie, A.G.W., Acta. Cryst. D52 (1996) 30-42) using an solvent fraction of 45%, resulting in a 2.1 Å electron density map that was interpretable. Refinement was performed by conjugate gradient and simulated annealing protocols as implemented in CNS 1.0 (Brünger, A.T., et al., Acta Crystallogr. D54 (1998) 905-921. All protocols included refinement of individual isotropic B-factors and using the amplitude based maximum likelihood target function. The R-factor dropped to 21.8 % (Rfree= 26.2 %, resolution range 16.2 – 2.1 Å) for the final model including 47 water molecules. The water model was calculated using ARP and verified by visual inspection. The final refinement statistics are shown in Table 8.

Table 8:

Statistics from the crystallographic analysis

	native	K_2PtCl_4
Resolution (Å)	16.2 – 2.1	18.6 – 2.5
Measurements	45345	32833
Unique measurements	8035	4925
% Complete (last shell/Å)	99.3 (96.9/2.23 – 2.11)	99.9 (95.4/2.64-2.5)
R_{sym} (%) (last shell)	8.2 (44.8)	8.8 (49.5)
$R_{Cullis\text{-}iso}$	-	0.77
P_{iso}	-	1.48
Res. for phase calc. (Å)	-	18.6 – 2.5
Mean FOM	-	0.41

Refinement statistics:

Resolution range (Å)	16.2 – 2.1
reflections in working set	7522
reflections in test set	501
R_{cryst} (%)	21.8
R_{free} (%)	26.2
Protein atoms (non-H)	765
Solvent atoms (non-H)	47
Average B-factor (Å^2)	38.1
r.m.s. ΔB (2Å cutoff)	3.4
Deviations from ideality (r.m.s.):	
Bond lengths (Å)	0.013
Bond angles (°)	1.7

$$R_{sym} = \frac{\sum |I(h)_i - \langle I(h) \rangle|}{\sum \langle I(h) \rangle}$$

$R_{Cullis\text{-}iso}$ = r.m.s. lack of closure / r.m.s isomorphous difference

P_{iso} (Phasing power) = $\langle |F_H| \rangle$ / r.m.s. lack of closure for all reflections

Mean FOM = mean figure of merit

R_{cryst} = Crystallographic R-factor for reflections used in refinement

R_{free} = Crystallographic R-factor for reflections not used in refinement

r.m.s. = Root mean square

Example 3

Determination of the binding affinity of IGFBP mutants

The IGF-binding properties of wildtype and mutant fragments and full-length IGFBPs were quantitatively analyzed by BIAcore biosensor measurements. BIAcore 2000, Sensor Chip SA and HBSwere obtained from BIAcore AB (Uppsala, Sweden). All experiments were performed at 25°C and HBS (20 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.5) was used as a running buffer and for the dilution of ligands and analytes. Biotinylated IGF-I was immobilized at a concentration of 5 nM and 10 nM in HBS at a flow rate of 5 µl/min to the strepavidin coated sensor chip resulting in signals of 40 and 110 resonance units (RU). Biotinylated IGF-II was immobilized at a concentration of 5 nM in HBS resulting in a signal of 20 RU. An empty flow cell was used as control for unspecific binding and bulk effects. The low ligand concentration was necessary to limit mass transport limitations and rebinding. For the same reason all kinetic experiments were performed at the highest possible flow rate of 100 µl/min. Each protein (wildtype and mutant IGFBPs or fragments of these proteins) was injected at four concentrations (250, 50, 10, and 2 nM). Each sample was injected for 2 min followed by dissociation in buffer flow for 4 min. After the dissociation phase the sensor chip was regenerated by injection of 10 µl 100 mM HCl at a flow rate of 5 µl/min. The kinetic parameters were calculated using the BIAevaluation 3.0 software (BIAcore AB). After subtraction of the blank sensorgram the kinetic rate constants were calculated from a general fit of an overlay of the sensorgrams of all concentration of one analyte using the method called "1:1 binding with mass transfer". IGF-I and IGF-II were biotinylated with a five-fold molar excess of D-biotinyl-ε-aminocaproic acid-N-hydroxysuccinimide ester using the reagents and the operation instructions of the Biotin Protein Labelling Kit (Roche Diagnostics GmbH, DE). After blocking with lysine, the reaction was dialyzed against 50 mM Na-phosphate, 50 mM NaCl, pH 7.5.

Example 4

Inhibition of IGF-I-induced IGF-IR phosphorylation by IGFBP mutants

Confluent monolayers of NIH3T3 cells stably expressing human IGF-IR in 3.5 cm dishes were starved in DMEM containing 0.5% dialyzed fetal calf serum. After 48 h, cells were incubated without any hormone or with 5 x 10^{-9} M IGF-I or 1 x 10^{-8} M IGF-II; each sample was preincubated with increasing concentrations of different IGF-binding proteins or fragments thereof at room temperature for 1 h. After a 10 min stimulation at 37°C, the medium was removed and cells were lysed with 250 µl of lysing buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P40, 1.5 mM $MgCl_2$, 1 mM EGTA (ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid, Aldrich, USA), 10 mM sodium orthovanadate, and protease inhibitor cocktail Complete (Roche Diagnostics GmbH, DE) for 10 min on ice. Subsequently, cells were scraped off the plate and the insoluble material was separated by centrifugation for 20 min at 4°C. The protein concentration of the supernatant was determined using the BCA kit from Pierce, Rockford, USA according to the manufacturer's instructions. Equal protein concentration was incubated with the SDS sample buffer (63 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, 0.05% bromphenolblue, 100 mM DTT), boiled for 5 min and loaded on a 7.5% SDS polyacrylamide gel. After electrophoresis the proteins were transferred on a nitrocellulose membrane which first was blocked for 1 h with the 3 % BSA containing PBST (phosphate buffered saline-Tween®), then overnight incubated with 1 µg/ml monoclonal anti-phosphotyrosine antibody 3-365-10 (Roche Diagnostics GmbH, DE) in PBST that contained 3% BSA. Unbound antibody was removed by extensive washing. The blot was then incubated with 1:10000 diluted anti-mouse IgG-specific antibody conjugated with horse raddish peroxidase (Roche Diagnostics GmbH, DE). The immunoblot was developed using the ECL kit from Amersham and the concentration of IGFBP which results in 50 % inhibition of the IGF-I receptor phosphorylation was determined.

Example 5

Determination of the inhibition of tumor cell growth by IGFBP mutants

MCF-7 cells (from ATCC, American type Culture Collection, Rockville, Maryland, U.S.A., HTB22) were used to investigate the inhibitory effect of IGFBP mutants on tumor cells. 1000 MCF-7 cells were seeded per well in medium containing 2.5 %

FBS (fetal bovine serum). The cells were cultured in the presence of various concentrations of IGFBPs for 48 h. The percentage of surviving cells was determined by MTT ((3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) assay and the concentration of binding protein which results in reduction of cell survival by 50 % was determined.

Example 6

Mutagenesis, expression and purification of mini-IGFBP-5s and subcloning of IGFBP-4 into Pet-28a (+)

6.1 Buffers and media

Cell growth media:

LB-medium	per 1 liter: peptone 10 g, yeast extract 5 g, NaCl 10 g, adjusted to pH 7.
LB-agar	per 1 liter: peptone 10 g, yeast extract 5 g, NaCl 10 g, bacto agar 15 g, adjusted to pH 7.
Minimal medium	per 1 liter: 0.5 g NaCl, 1 g citric acid monohydrate, 36 mg ferrous citrate (pre-dissolved in conc. HCl), 4.02 g KH_2PO_4, 7.82 g K_2HPO_4, 1g ^{15}N-NH_4Cl, 1.3 ml trace elements solution (per liter of the stock solution: 2.5 g H_3BO_3, 2.0 g $CoCl_2$, 1.13 g $CuCl_2$, 9.8 g $MnCl_2$, 2.0 g Na_2MoO_4), 1 ml Zn-EDTA solution (per ml of the stock solution: 5 mg EDTA, 8.4 mg zinc acetate), adjusted to pH 7, autoclaved. Added afterwards: 25 ml autoclaved 20% (w/v) glucose, 560 µl sterile filtered 1% (w/v) thiamine, 2ml 1M $MgSO_4$.

Antibiotic stocks:

Ampicillin	50 mg/ml in dist. water, 0.22 µm filtrated, stored at –20°C.
Kanamycin	25 mg/ml in dist. water, 0.22 µm filtrated, stored at –20°C.
Chloramphenicol	35 mg/ml in 96 % ethanol, stored at –20 °C.

Agarose-gel electrophoresis:

TAE-buffer (50x)	2 M Tris-HCl (pH 8.0), 2 M glacial acetic acid and 50 mM EDTA.
Loading buffer (3x)	0.13 % bromophenol blue, 0.13 % xylene cyanol, 30 % glycerol.
Et-Br-solution	10 mg/ml ethidiumbromide in dd H_2O.

SDS-PAGE:

Sample buffer (5x)	125 mM Tris-HCl (pH 6.8), 10 % SDS, 760 mM 2-mercaptoethanol, 0.13 % bromophenol blue, 50 % glycerol and 2 mM EDTA.
Staining solution	0.125 % CBB-R250 in 500 ml 96 % ethanol and 500 ml 10 % acetic acid.
Distaining solution	96 % ethanol, 10 % acetic acid and dest. H_2O in 4:3:3 proportion.

Tricine gels:

Cathode (top) running buffer (10x)	1 M Tris-HCl (pH 8.25), 1 M Tricine and 1 % SDS.
Anode (bottom) running buffer (10x)	2 M Tris-HCl (pH 8.9).
Separation buffer	3 M Tris-HCl (pH 8.9) and 0.3 % SDS.
Stacking buffer	1 M Tris-HCl (pH 6.8) and 0.3 % SDS.
Separation acrylamide	48 % (w/v) acrylamide, 1.5 % (w/v) N,N'-methylene-bis-acrylamide.
Stacking acrylamide	30 % (w/v) acrylamide, 0.8 % (w/v) N,N'-methylene-bis-acrylamide.
APS	10 % ammonium persulphate in dd H_2O.
Separation gel (main)	for 2 70x80x0.75 mm mini-gels: 1.675 ml H_2O, 2.5 ml separation buffer, 2.5 ml separation acrylamide, 0.8 ml glycerol, 25 µl APS and 2.5 µl TEMED.

Separation gel (intermediate)	1.725 ml H_2O, 1.25 ml separation buffer, o.75 ml separation acrylamide, 12.5 µl APS and 1.25 µl TEMED.
Stacking gel	2.575 ml H_2O, 0.475 ml stacking buffer, 0.625 ml stacking acrylamide, 12.5 µl 0.5 M EDTA (pH 8.0), 37.5 µl APS and 1.9 µl TEMED.

Protein purification:

Buffer A	6 M guanidinium-HCl, 100 mM NaH_2PO_4, 10 mM Tris and 10 mM 2-mercaptoethanol, pH 8.0.
Buffer B	6 M guanidinium-HCl, 100 mM NaH_2PO_4, 10 mM Tris and 10 mM 2-mercaptoethanol, pH 6.5
Buffer C	6 M guanidinium-HCl, 100 mM Na-acetate and 10 mM 2-mercaptoethanol, pH 4.0.
Buffer D	6 M guanidinium-HCl, pH 3.0.
Buffer E	200 mM arginine, 1 mM EDTA, 100 mM Tris-HCl, 2 mM reduced glutathione, 2 mM oxidised glutathione, pH 8.4.
PB(0)	10 mM Na_2HPO4, 1.8 mM KH_2PO_4 and 0.05 % NaN_3, pH 7.2.
PB(1000)	10 mM Na_2HPO4, 1.8 mM KH_2PO_4, 0.05 % NaN_3 and 1 M NaCl, pH 7.2.
PBS	140 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 and 0.05% NaN_3.
Thrombin cleavage buffer	60 mM NaCl, 60 mM KCl, 2.5 mM $CaCl_2$, 50 mM Tris, pH 8.0.

6.2 Cloning of mini-IGFBP-5

Mini-IGFBP-5 (residues 40-92 of IGFBP-5) was subcloned from a vector containing the complete sequence of IGFBP-5 into the BamHI and PstI restriction sites of the pQE30-vector (Qiagen, Hilden, Germany). Restriction sites, a stop codon and 21 bases encoding an N-terminal thrombin cleavage site were introduced by means of PCR (Kalus, W., et al., EMBO J. 17 (1998) 6558-6572).

6.3 Mutagenesis of mini-IGFBP-5

For introduction of mutations leading to substitution of Leu_{61} by Tyr and Leu_{74} by Met, in vitro mutagenesis was performed using QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, Canada). Two sets of the following mutagenic oligonucleotide primers were designed for amplification of plasmid DNA and introduction of the desired point mutations:

FBP5LY: 5'-G GGG CTG CGC TGC TAC CCC CGG CAG GAC G-3';
(SEQ ID NO:1)
RBP5LY: 5'-C GTC CTG CCG GGG GTA GCA GCG CAG CCC C-3';
(SEQ ID NO:2)
FBP5LM: 5'-CG CTG CAC GCC CTG ATG CAC GGC CGC GGG G-3';
(SEQ ID NO:3)
RBP5LM: 5'-C CCC GCG GCC GTG CAT CAG GGC GTG CAG CG-3'
(SEQ ID NO:4).

The changed codons (CTC into TAC in $L_{61}Y$ mutant and CTG into ATG in $L_{74}M$ mutant) are presented in bold. Degenerated bases are underlined.

The reactions were set up according to the instructions found in the mutagenesis kit manual. The PCR mixtures (50 µl) contained app 50 ng of the template (pQE30 (mini-IGFBP-5), prepared by means of mini prep spin columns kit, Qiagen) and 125 ng of each of the two oligonucleotide primers. Cycling parameters for the reactions were as follows: 30 seconds at 95°C followed by 13 cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 7.5 min. The DpnI digestion and XL1-Blue supercompetent cells transformation was carried out strictly according to the supplier's guidelines.

Two clones of each mutant were subjected to verification by automated double stranded sequencing, which proved the existence of the expected substitutions in all 4 cases.

6.4 Expression of the mutant mini-IGFBP-5s

Electrocompetent cells BL21 were transformed with the construct carrying the mutation. From a fresh plate, a 3-ml LB culture was started and grown overday (6-7 h) in the presence of 300 µg ampicillin per ml at 37°C. From this culture 50 µl were used to inoculate 20 ml of MM. This culture was grown overnight (9-11h). 1 l culture was inoculated in 1:50 proportion. Expression of the protein was induced at $OD_{600} \cong 0.8$ by addition of IPTG (1 mM final concentration). Cells were harvested after 3 h (6000 xG, 20 min at 4°C).

6.5 Purification of mini-IGFBP-5

Harvested cells were resuspended in buffer A (30 ml of the buffer was used to resuspend cells from 1l culture) and incubated at room temperature with vigorous shaking (280 RMP) for 1 h to overnight. The cells were opened by sonification (macrotip, 50 % duty cycle, output control 70, 2x4 min). The cell extract was then centrifuged to pellet cell debris (65 000 xG, 1h at room temp.). The pH of the supernatant was adjusted to the value of app. 8.0. The supernatant was then mixed with pre-equilibrated with buffer A Ni-NTA Superflow matrix (Qiagen) , incubated with agitation for 1 h to overnight and then loaded onto an empty column (3 ml bed volume for 1 l culture). The column was washed with buffer A followed by buffer B until a stable UV-absorption base line. Bound proteins were fractionated with 100 ml pH gradient of buffer B and C. Collected fractions were analysed by tricine gel electrophoresis (prior electrophoresis, the proteins were precipitated with 5 % (w/v) TCA). Fractions containing mini-IGFBP-5 were pooled, concentrated on Amicon YM3 to 2-4 ml, and dialysed against 2 l of buffer D overnight (100 µl excess of 2-mercaptoethanol was added to the sample prior dialysis).

To promote refolding, the dialysed sample was diluted in 100 µl portions into freshly prepared, ice-cold buffer E, with vigorous stirring (in proportion 1 ml sample per 50 ml of buffer E), and left at 4°C for 2-3 days with stirring.

The sample was concentrated on Amicon YM3 to 15-25 ml, centrifuged to get rid of a precipitated material, and dialysed overnight into 4 l of buffer PB containing 30 mM NaCl.

The solution was subsequently loaded onto pre-equilibrated with buffer PB (0) MonoS 5/5 HR cation-exchanger column (app. 1 ml) (Amersham Pharmacia, Uppsala, Sweden) at a flow rate of 1 ml/min. The column was washed with buffer PB (0). Proteins were eluted by 45 ml linear gradient of 0-70 % NaCl, 1 ml fractions were collected.

The fractions containing mini-IGFBP-5 (as determined on the basis of tricine gel electrophoresis) were pooled, concentrated to 2-3 ml and loaded onto a pre-equilibrated with PBS Superdex 75 HiLoad 26/60 (app. 320 ml) gel-filtration column (Pharmacia) at a flow rate of 0.6 ml/min. Mini-IGFBP-5 was eluted as a symmetrical, single pick. Fractions containing the protein were pooled and concentrated on centricon YM3.

6.6 Subcloning into pET-28a (+)

The reason for overall low expression of the proteins from the pQE30 might be the fact that this vector is not well optimised for expression in E. coli. For instance, the vector-encoded sequences contain a cluster of 3 rare codons just downstream from the initiator codon AUG (namely, AGA, GGA and TCG, encoding Arg, Gly and Ser, respectively). The number of studies has indicated that excessive rare codon usage in a target gene may be a cause for low level expression. The impact seems to be most severe when multiple rare codons occur near the amino terminus and when they appear consecutively. Especially presence of the Arg codons AGG and AGA can have severe effects on the level of protein production. The system seems to be also not well repressed (no extra copies of a gene encoding Lac repressor), and the leaky expression may cause the observed plasmid instability. The vector carries not very efficient selective marker, Amp^R gene (bla), what makes possible rapid over-growing of a culture at a certain stage by cells lacking the unstable plasmid. The vector pET-28a (+) (Novagen) was then chosen as an alternative for pQE30. The plasmid is well optimised for expression of genes in E. coli, carries a strong selective marker (Kan^R) and is stable due to high level of repression of the target gene expression in the absence of IPTG (in a non-DE3 lysogenic strain even in the presence of the inducer).

To subclone mini-IGFBP-5 wild type, $L_{61}Y$ and $L_{74}M$ from pQE30 to pET-28a, the relevant fragments were excised from the vector with BamHI and HindIII (HindIII

cleavage site exists in pQE30 downstream from PstI site). The excision was performed as double-digestion. Digested pET vector was 5'-dephosphorylated. Reaction mixtures were electrophorized and bands corresponding to app. 200 bp fragments excised from pQE30 (mini-IGFBP-5 wt, $L_{61}Y$ and $L_{74}M$) and app 5000 bp fragment of pET-28a were cut from 1 % agarose gel and purified (gel extraction kit, Qiagen). The fragments were ligated (Ligation kit, Fermentas) and XL-1 Blue Supercompetent cells were transformed with the ligation mixture.

Restriction assay carried out subsequently on isolated plasmid DNA revealed presence of fragments of expected size (restriction enzymes NcoI and PstI were used, double digestion was performed. PstI restriction site was introduced into the pET vector together with the fragment encoding mini-IGFBP-5).

Pilot-scale expression and purification experiment showed that expression of the protein of interest (mini-IGFBP-5 $L_{61}Y$ in this case) is higher than the expression of the wild-type protein when pQE30 vector was used.

The proteins are expressed as double-fusions: they carry His-tag followed by T7-tag. It is possible to remove both tags by thrombin cleavage. Mini-IGFBP-5 after cleavage by thrombin comprises the following N-terminal amino acid sequence: GSALA (SEQ ID NO:7) (N-terminus of mini-IGFBP-5 starting from aa 40 with to additional aa from cloning with thrombin cleavage site). Vector-derived amino acids are underlined.

6.7 Subcloning of IGFBP4 from pKK177-3HB to pET-28a(+)

For subcloning of IGFBP4-2 into the NdeI and BamHI restriction sites of the pET-28a vector in-frame to a His-tag, following oligonucleotides were designed for amplification of DNA by PCR:

IBP4NdeI: 5'-CGG AGG AAA AAC ATA TGG ATG AAG C-3'
(SEQ ID NO:5)
IBP4BamHI: 5'-GCC AAG CTT GGA TCC AGG TCG AC-3'
(SEQ ID NO:6)

The restriction sites recognized by NdeI and BamHI are presented in bold. Degenerated bases are underlined.

The PCR mixture (50 µl) contained 124 ng of mixture of pKK177-3HB and Pfdx500 repressor plasmid, 130 ng of each of the primers, 1 µl dNTP mix and 2.5 U Pfu Turbo DNA polymerase (Strategene). After initial step of 30 sec. At 95°C, the reaction was cycled 30x at 95°C for 30 seconds, 55°C for 1 min and 68°C for 2 min. The product of PCR was purified (PCR purification kit, Qiagen), double-digested and electrophorised. The bands corresponding to cleaved pET-28a and PCR product were excised from the gel and purified.

XL-1 Blue Supercompetent cells were transformed with the ligation mixture.

IGFBP4-2 is expressed as a N-terminal His-tag fusion protein. After thrombin cleavage, the protein comprises the following amino acid sequence: GSHMDEAIH... (SEQ ID NO:8). Vector derived amino acids are underlined.

The same purification routine will be used for His-tagged IGFBP-4 as for mini-IGFBP-5.

Example 7

Identification of chemical non-proteinaceous compounds binding to IGFBP-5 or IGF-I by using the coordinates of the crystal structure of the complex of both molecules

FlexX version 1.9.0 was used to screen a substance library of ca. 90,000 compounds in an ACD (Available Chemicals Directory; ACD-3D 2000), choosing compounds with a molecular weight of less than 550 Daltons and containing at least one of the atoms {N, O, F, or S}. Docking searches were conducted on both molecular surfaces of the IGFBP-5 interface. Top scoring hits as judged by the FlexX standard scoring function and the proximity to binding site protein atoms were selected and tested for activity.

The top scoring compounds selected according to these these criteria for release of IGF-I from IGFBP-5 were:

Compound 1: N1-(3,4-Dichlorophenyl)-2-[2-[5-(3,5-dichlorophenyl)-2H-1,2,3,-tetraazol-2YL]A (MF: C16H11Cl4N7OS; MW: 491,1890 Da)

Compound 2: F-MOC-Tyr(PO3H2)-OH (C24H22NO8P; MW: 483.4110)
Compound 2A: Nα-FMOC-O-tert-butyl-L-tyrosine
Compound 2B: Nα-FMOC-L-phenylalanine
Compound 2C: Nα-FMOC-N-BOC-L-tryptophan
Compound 2D: Nα-FMOC-L-leucine

Compound 3: 4-(2,5-Dichlorophenylazo)-4`fluorosulfonyl-1-hydroxy-2-naphthanilide (MF: C23H14Cl2FN3O4S; MW: 518.3510)

Compound 4B: 5-Amino-2[(4-amino-2-carboxyphenyl)thio]benzoic acid (C14H12N2O4S; MW 304.3250)

Compound 4C: 5-Amino-2[(2-carboxyphenyl)thio]benzoic acid (C14H11NO4S; MW 289.3100)

Example 8

Release of IGF-I from the complex with IGFBP-5 by selected compounds measured by IGF-I binding to IGF-IR expressing cells

Kalus, W., et al., in EMBO J. 17 (1998) 6558-6572, describe the inhibition of the binding of IGF-I to IGF-IR expressing NIH 3T3 cells by formation of an inhibitory complex. This assay was used to investigate the release of IGF-I from the inhibitory complex with IGFBP-5.

NIH 3T3 cells stably expressing human IGF-IR were grown in culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cells were washed carefully with PBS and incubated with 5 ml of 50 mM EDTA in PBS for 45 min. Cells were removed from the plate, washed once with PBS and once with binding buffer (100 mM HEPES pH 7.6, 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO 4 , 1 mM EDTA, 10 mM glucose, 15 mM sodium acetate, 1% dialysed BSA), and resuspended in binding buffer to determine the cell number. 5 pM ^{125} I-IGF-I (Amersham) was preincubated with either 10 or 100 nM IGFBP-5 alone or in

combination with 33 µM of the different compounds 1,2,3,4B and 4C at 4°C for 1 h. Then 400 µl of the cell suspension corresponding to $2x10^5$ cells were added to give a total volume of 500 µl. After 12 h incubation at 4°C, cells were washed with binding buffer (at 4°C). Free hormone was removed by repeated centrifugation and resuspension in the binding buffer. The 125 I radioactivity bound to the cells was determined in a gamma-counter.

As shown in figure 7 the labeled IGF-I binds to NIH 3T3 cells in the absence of IGFBP-5 and cell binding is inhibited by the addition of IGFBP-5. Preincubation of the complex of IGFBP-5 and IGF-I with the selected compounds results in release of IGF-I from the complex by compound 3 and consequently binding of IGF-I to the IGF-IR expressing cells.

Example 9

Release of IGF-I from the complex with IGFBP-5 by selected compounds measured by IGF-IR activation

Kalus, W., et al., in EMBO J. 17 (1998) 6558-6572, describe the inhibition of the activation and autophosphorylation of the IGF-IR by IGF-I in the presence of IGFBP-5. This assay was used to further investigate the release of IGF-I from the inhibitory complex with IGFBP-5 by compound 3. Binding of compound 3 to IGFBP-5 and dissociation of the complex of the binding protein with IGF-I should result in an activation and autophosphorylation of the IGF-IR in the presence of IGFBP-5.

Confluent monolayers of the NIH 3T3 cells stably expressing human IGF-IR in 3.5 cm dishes were starved in DMEM containing 0.5% dialysed fetal calf serum. After 48 h, cells were incubated without any hormone or with 10 nM IGF-I. Samples were preincubated with 100 nM IGFBP-5 and increasing concentrations of compound 3 from 0 to 50 µM at room temperature for 1 h. After a 10 min stimulation at 37°C, the medium was removed and cells were lysed with 250 µl of lysing buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 1.5 mM MgCl 2 , 1 mM EGTA), 10 mM sodium orthovanadate, and protease inhibitor cocktail Complete (Roche Molecular Biochemicals) for 10 min on ice. Subsequently, cells were scraped off the plate and the insoluble material was separated by centrifugation for 20 min at 4°C. The protein concentration of the

supernatant was determined using the BCA kit from Pierce according to the manufacturer's instructions. Equal protein concentration was incubated with the SDS sample buffer (63 mM Tris–HCl pH 6.8, 3% SDS, 10% glycerol, 0.05% bromophenolblue, 100 mM DTT), boiled for 5 min and loaded on a 7.5% SDS–polyacrylamide gel. After electrophoresis the proteins were transferred on a nitrocellulose membrane which first was blocked for 1 h with the 3% BSA containing phosphate-buffered saline–Tween (PBST), then incubated overnight with 1 mg/ml monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology), polyclonal anti-phospho-AKT antibody (New England Biolabs) or polyclonal anti- IGF-IR (C-20, Santa Cruz Biotechnology) in PBST that contained 3% BSA. Unbound antibody was removed by extensive washing. The blot was then incubated with 1:10 000 diluted anti-mouse IgG-specific antibody or 1:5000 diluted anti-rabbit specific antibody conjugated with horse radish peroxidase (both Roche Molecular Biochemicals). The immunoblot was developed using the ECL kit from Amersham.

As shown in Fig. 8 the autophosphorylation of IGF-IR by IGF-I is inhibited in the presence of IGFBP-5. The addition of compound 3 to the inactive complex of IGFBP-5 and IGF-I results in an increased autophosphorylation of the receptor at 50 uM compound 3.

Example 10

Detection of ligand binding

Ligand binding was detected by acquiring ^{15}N-HSQC spectra. All NMR spectra were acquired at 300 K on Bruker DRX600 spectrometer. The samples for NMR spectroscopy were concentrated and dialyzed against PBS buffer. Typically, the sample concentration was varied from 0.3 to 1.0 mM. Before measuring, the sample was centrifuged in order to sediment aggregates and other macroscopic particles. 450 µl of the protein solution were mixed with 50 µl of D_2O (5-10%) and transferred to an NMR sample tube. The stock solutions of compounds were 100 mM either in water or in perdeuterated DMSO. pH was maintained constant during the whole titration. The binding was monitored by observation of the changes in the ^{15}N-HSQC spectrum. Dissociation constants were obtained by monitoring the chemical shift changes of the backbone amide of several amino acid residues (Table 9) as a function of ligand concentration. Data were fit using a single

binding site model. In the same way dissociation constants for derivatives of compound 2 are estimated (Table 10).

Table 9:

Dissociation constant calculations for compound 2 or DMSO binding to IGFBP-5 using data from distinct amino acid residues

residue	ligand in DMSO K_D [mM]	ligand in PBS K_D [mM]	DMSO K_D [mM]
Y50	1.58 ± 0.09	1.82 ± 0.95	648 ± 370
L73	1.31 ± 0.17	2.93 ± 1.41	541 ± 306
S85	1.38 ± 0.10	2.33 ± 0.94	650 ± 373
Y86	1.90 ± 0.17	1.72 ± 0.99	783 ± 498
R87	1.64 ± 0.12	2.36 ± 1.00	921 ± 662
K91	2.42 ± 0.18	2.12 ± 1.03	719 ± 434
average:	1.71 ± 0.37	2.21 ± 0.40	710 ± 120

Table 10:

Dissociation constants calculated for compound 2 and its derivatives binding to IGFBP-5 using changes in chemical shift for the residue L81

compound	chemical name	K_D [mM]
2	Nα-FMOC-O-phospho-L-tyrosine	2.78 ± 0.30
2A	Nα-FMOC-O-tert-butyl-L-tyrosine	0.718 ± 0.079
2B	Nα-FMOC-L-phenylalanine	1.075 ± 0.507
2C	Nα-FMOC-N-BOC-L-tryptophan	0.0432 ± 0.0115
2D	Nα-FMOC-L-leucine	1.088 ± 0.519

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Patent Claims

1. A crystal suitable for X-ray diffraction, comprising a complex of insulin-like growth factor I or II (IGF) and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40–92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9^{th} to 12^{th} cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7^{th} to 10^{th} cysteine of IGFBP-6, to form a complex which exhibits restricted conformation mobility.

2. A crystal of claim 1, which effectively diffracts X-ray for the determination of the atomic coordinates of the complex to a resolution of 1.5 to 3.5 Å.

3. A crystal of claim 1 or 2, wherein the crystal is arranged in the cubic space group $P2_13$ having unit cell dimensions of 74.385 Å. x 74.385 Å x 74.385 Å.

4. A method for producing a crystal suitable for X-ray diffraction, comprising

   (a) contacting IGF with a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40–92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9^{th} to 12^{th} cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7^{th} to 10^{th} cysteine of IGFBP-6, to form a complex which exhibits restricted conformation mobility, and

   (b) obtaining a crystal from the complex so formed suitable for X-ray diffraction.

5. A method for the determination of the atomic coordinates of a crystal suitable for X- ray diffraction obtained by

   (a) contacting IGF with a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99

of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40–92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, to form a complex which exhibits restricted conformation mobility; and

(b) obtaining a crystal from the complex so formed suitable for X-ray diffraction;

(c) determining the atomic coordinates of said crystal.

6. A method for identifying a mutant of IGFBP (IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 or IGFBP-6 or a mutant of a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6) having an enhanced binding affinity for IGF, comprising

(a) constructing a three-dimensional structure of the complex of IGF and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40–92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, based on the atomic coordinates of a crystal consisting of IGFI and said IGFBP or a fragment thereof;

(b) employing said three-dimensional structure and modeling methods to identify said mutant of an IGFBP in which a residue within a distance of 5 Å to a hydrophobic amino acid residue of IGF is modified in that the hydrophobic interaction between IGF and said mutant of IGFBP is enhanced;

(c) producing said mutant;

(d) assaying said mutant to determine said enhanced binding affinity for IGF.

7. A method for identifying a mutant of IGFBP-5 with enhanced binding affinity for IGF-I, said method comprising

(a) constructing a three-dimensional structure of the complex of IGF and IGFBP-5 defined by the atomic coordinates shown in Figs. 5 and 6;

(b) employing said three-dimensional structure and modeling methods to identify an amino acid residue in IGFBP-5 within a distance of 5 Å or shorter to an amino acid residue of IGFI, wherein said residue of IGFBP-5 can be modified hydrophobically in that the hydrophobic interaction between IGF and IGFBP-5 is enhanced;

(c) producing said mutant;

(d) assaying said mutant to determine said enhanced binding affinity for IGF.

8. A mutant of IGFBP containing one or more of the mutations as depicted in Tables 1 to 6.

9. A mutant of IGFBP containing one or more mutations of amino acid residues 49, 70 and/or 73 corresponding to IGFBP-5 sequence alignment according to Tables 1 to 6.

10. A method for identifying a non-proteinaceous compound capable of binding to IGFBP, comprising

(a) constructing a three-dimensional structure of a complex of insulin-like growth factor I or II and a polypeptide consisting of the amino acids 40-92 of insulin-like growth factor binding protein 5, amino acids 39-91 of IGFBP-1, amino acids 55-107 of IGFBP-2, amino acids 47-99 of IGFBP-3, amino acids 39-91 of IGFBP-4, amino acids 40-92 of IGFBP-5, amino acids 40-92 of IGFBP-6 or a fragment thereof

consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, based on the atomic coordinates of a crystal consisting of IGF – I and said IGFBP;

(b) employing said three-dimensional structure and modeling methods to identify a non-proteinaceous compound forming a complex with said IGFBP by hydrophobic binding with amino acids 49, 50, 70, 71 and 74 in the case of IGFBP-5 and in the case of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-6 with the corresponding amino acids according to Table 7;

(c) producing said compound;

(d) determining the binding between the compound and said IGFBP.

Fig. 1A

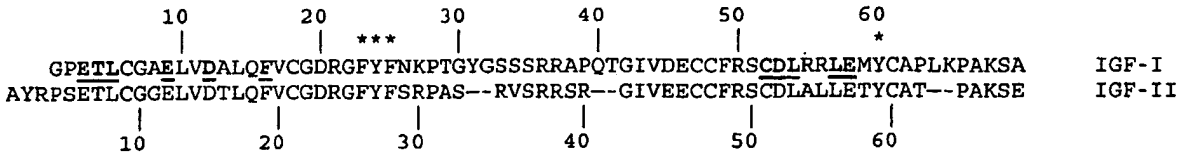

Fig. 1B

```
P08833    BP1    --QVGVTAG---APWQCAPCSAEKLALCPP----VSASCS----------EVTRSAGCG
P18065    BP2    ---GGGARA--EVLFRCPPCTPERLAACGPPPVAPPAAVAAVAGGARMPCAELVREPGCG
P17936    BP3    ---AGASSGGLGPVVRCEPCDARALAQCAPP----PAVCA----------ELVREPGCG
P22692    BP4    LLAAGPGPSLGDEAIHCPPCSEEKLARCRP-----PVGCE----------ELVREPGCG
P24593    BP5    --YAGPAQS-LGSFVHCEPCDEKALSMCPPS----PLGC----------ELVKEPGCG
P24592    BP6    ---LAASPG--GALARCPGCGQGVQAGC-------PGGCVEE---------EDGGSPAEG
                        .   .       :*  *     : *      .                *   ... *
consensus/100%   ...huss.u.....hpC.sCs.th.u.C.......shss............E.stpsupG

                                 40        50        60        70        80
                                 |         |         |         |         |
                            GSmmmmm(mini-BP)mmmmmmmmmmmmmmmmmmmmmmmmmmmmmmmmmm--mm----m
P08833    BP1    CCPM--CALPLGAACGVATARCARGLSCRALPGEQQPLHALTRGQGACVQE---SD---A
P18065    BP2    CCSV--CARLEGEACGVYTPRCGQGLRCYPHPGSELPLQALVMGEGTCEKR---RDAEYG
P17936    BP3    CCLT--CALSEGQPCGIYTERCGSGLRCQPSPDEARPLQALLDGRGLCVNASAVSRLRAY
P22692    BP4    CCAT--CALGLGMPCGVYTPRCGSGLRCYPPRGVEKPLHTLMHGQGVCMEL-----A---
P24593    BP5    CCMT--CALAEGQSCGVYTERCAQGLRCLPRQDEEKPLHALLHGRGVCLNE--KS----Y
P24592    BP6    CAEAEGCLRREGQECGVYTPNCAPGLQCHPPKDDEAPLRALLLGRGRCLPA---R-----
                 *.    *    *  **: * .*. ** * .  .   **::*  *.* *
consensus/100%   Cs.h..Chh..G..CGlhT.pCu.GLpC.s..s.t.PLpsLh.GpGhC...........

                 mmmmmm
P08833    BP1    SAPHAAEAGSP-ESPES--TEIT
P18065    BP2    ASPEQVAD-NGDDHSEGGLVENH
P17936    BP3    LLPAPPAPGNASESEEDRSAGSV
P22692    BP4    EIEAIQESLQPSDKDEGDHPNNS
P24593    BP5    REQVKIER-DSREHEEPTTSEMA
P24592    BP6    -APAVAEE-NPKESKPQ--AGTA
                        .  :
consensus/100%   ......t..ps.-p.....st..
```

Fig. 2

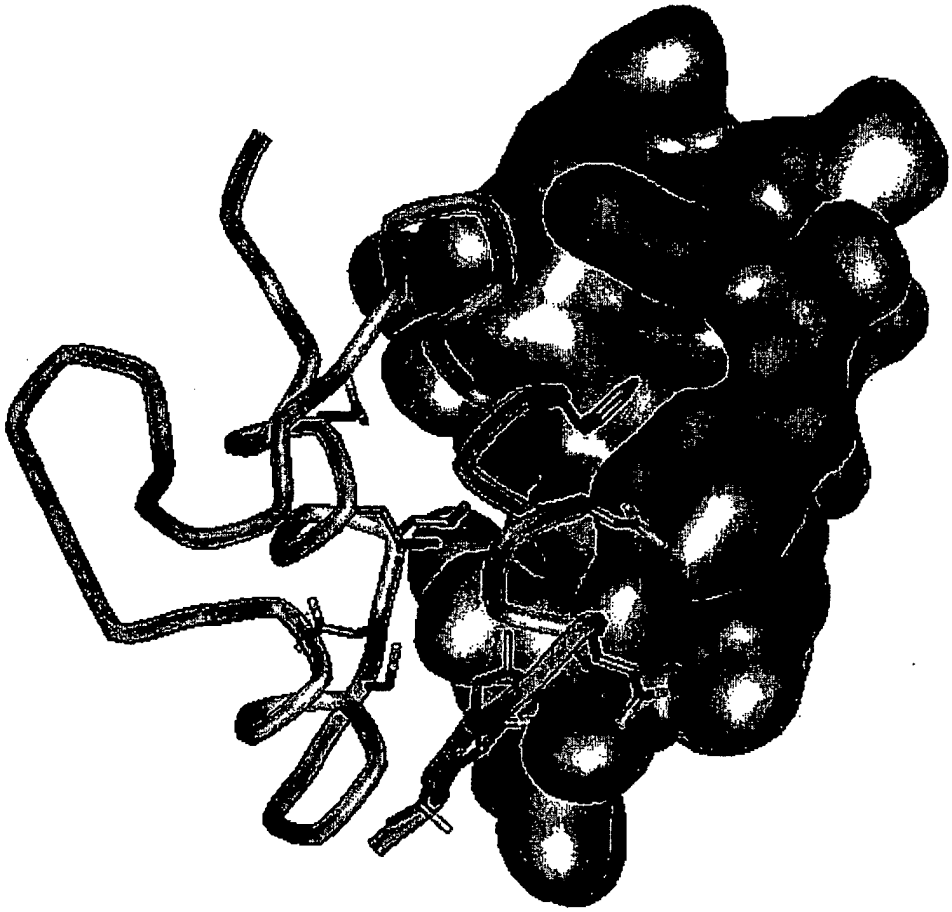

Fig. 3

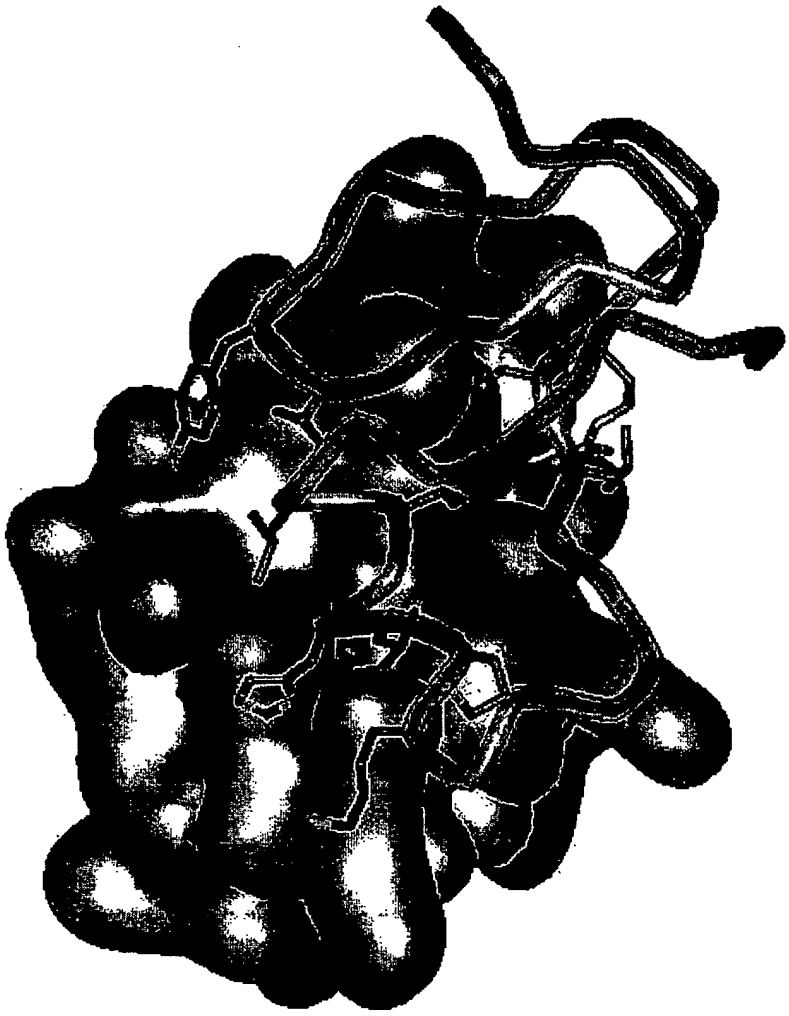

Fig. 4A

	IGF-BP5 Residues										
	Gly48	Val49	Tyr50	Cys60	Glu67	Lys68	Pro69	Leu70	His71	Leu73	Leu74
IGF-Glu3						5	2.9	3.2	3.1	2.5		
IGF-Thr4										3.3		
IGF-Leu5									4.0	4.1		3.7
IGF-Glu9				4.9						4.5		3.9
IGF-Asp12			4.0	2.6								4.8
IGF-Ala13			4.8									4.8
IGF-Ph 16	3.5	3.7			3.7						4.6	

Fig. 4B

	IGF-BP5 residues				
	Tyr50	Lys68	Pro69	Leu70	His71
IGF-Glu3		SC-SC CHB	SC-SC CHB	SC-MC CHB	SB, SC-MC HB
IGF-Thr4					MC-SC CHB
IGF-Asp12	SC-SC HB				

Fig. 5

```
ATOM      1  CB  PRO A   2      10.704  22.686  21.862  1.00 48.41      A
ATOM      2  CG  PRO A   2      11.649  23.436  20.928  1.00 49.62      A
ATOM      3  C   PRO A   2       9.908  20.943  20.252  1.00 44.31      A
ATOM      4  O   PRO A   2       9.908  21.594  19.208  1.00 46.80      A
ATOM      5  N   PRO A   2      12.286  21.170  20.867  1.00 48.45      A
ATOM      6  CD  PRO A   2      12.881  22.517  20.805  1.00 49.31      A
ATOM      7  CA  PRO A   2      10.895  21.262  21.370  1.00 47.18      A
ATOM      8  N   GLU A   3       9.065  19.942  20.475  1.00 40.09      A
ATOM      9  CA  GLU A   3       8.078  19.540  19.478  1.00 35.47      A
ATOM     10  CB  GLU A   3       7.425  18.219  19.904  1.00 31.82      A
ATOM     11  CG  GLU A   3       6.324  17.741  18.977  1.00 30.50      A
ATOM     12  CD  GLU A   3       5.811  16.350  19.321  1.00 29.25      A
ATOM     13  OE1 GLU A   3       5.562  16.074  20.512  1.00 29.70      A
ATOM     14  OE2 GLU A   3       5.640  15.537  18.391  1.00 28.06      A
ATOM     15  C   GLU A   3       7.002  20.607  19.258  1.00 33.04      A
ATOM     16  O   GLU A   3       6.553  21.252  20.198  1.00 33.26      A
ATOM     17  N   THR A   4       6.605  20.796  18.006  1.00 33.52      A
ATOM     18  CA  THR A   4       5.564  21.761  17.671  1.00 32.64      A
ATOM     19  CB  THR A   4       6.133  23.010  16.949  1.00 33.01      A
ATOM     20  OG1 THR A   4       6.850  22.600  15.781  1.00 34.92      A
ATOM     21  CG2 THR A   4       7.063  23.795  17.868  1.00 33.39      A
ATOM     22  C   THR A   4       4.539  21.099  16.756  1.00 32.21      A
ATOM     23  O   THR A   4       4.854  20.143  16.045  1.00 31.41      A
ATOM     24  N   LEU A   5       3.311  21.607  16.794  1.00 28.70      A
ATOM     25  CA  LEU A   5       2.225  21.094  15.973  1.00 27.95      A
ATOM     26  CB  LEU A   5       1.248  20.263  16.811  1.00 30.16      A
ATOM     27  CG  LEU A   5       1.261  18.739  16.718  1.00 32.01      A
ATOM     28  CD1 LEU A   5      -0.021  18.204  17.364  1.00 27.14      A
ATOM     29  CD2 LEU A   5       1.346  18.295  15.262  1.00 28.94      A
ATOM     30  C   LEU A   5       1.479  22.281  15.380  1.00 27.71      A
ATOM     31  O   LEU A   5       1.089  23.196  16.102  1.00 25.30      A
ATOM     32  N   CYS A   6       1.274  22.257  14.068  1.00 27.68      A
ATOM     33  CA  CYS A   6       0.586  23.344  13.387  1.00 27.97      A
ATOM     34  C   CYS A   6      -0.467  22.815  12.429  1.00 27.16      A
ATOM     35  O   CYS A   6      -0.555  21.617  12.189  1.00 25.39      A
ATOM     36  CB  CYS A   6       1.580  24.177  12.571  1.00 31.40      A
ATOM     37  SG  CYS A   6       3.038  24.809  13.457  1.00 38.95      A
ATOM     38  N   GLY A   7      -1.255  23.736  11.887  1.00 26.22      A
ATOM     39  CA  GLY A   7      -2.279  23.399  10.914  1.00 25.51      A
ATOM     40  C   GLY A   7      -3.307  22.330  11.229  1.00 25.84      A
ATOM     41  O   GLY A   7      -3.814  22.213  12.351  1.00 24.26      A
ATOM     42  N   ALA A   8      -3.633  21.559  10.199  1.00 22.75      A
ATOM     43  CA  ALA A   8      -4.622  20.495  10.301  1.00 23.80      A
ATOM     44  CB  ALA A   8      -4.783  19.804   8.946  1.00 23.28      A
ATOM     45  C   ALA A   8      -4.242  19.484  11.371  1.00 22.59      A
ATOM     46  O   ALA A   8      -5.102  18.969  12.073  1.00 23.77      A
ATOM     47  N   GLU A   9      -2.950  19.205  11.511  1.00 23.09      A
ATOM     48  CA  GLU A   9      -2.528  18.244  12.522  1.00 24.67      A
ATOM     49  CB  GLU A   9      -1.047  17.911  12.370  1.00 26.80      A
ATOM     50  CG  GLU A   9      -0.741  17.101  11.132  1.00 32.24      A
ATOM     51  CD  GLU A   9       0.698  16.651  11.096  1.00 36.38      A
ATOM     52  OE1 GLU A   9       1.588  17.526  11.148  1.00 39.56      A
ATOM     53  OE2 GLU A   9       0.936  15.427  11.022  1.00 37.49      A
ATOM     54  C   GLU A   9      -2.799  18.730  13.935  1.00 21.39      A
ATOM     55  O   GLU A   9      -3.115  17.934  14.812  1.00 21.62      A
ATOM     56  N   LEU A  10      -2.660  20.032  14.163  1.00 20.36      A
ATOM     57  CA  LEU A  10      -2.915  20.588  15.484  1.00 19.60      A
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Fig. 5 (contd.)

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ATOM     58  CB  LEU A  10      -2.488  22.061  15.530  1.00 20.83      A
ATOM     59  CG  LEU A  10      -2.900  22.790  16.813  1.00 23.06      A
ATOM     60  CD1 LEU A  10      -2.279  22.087  18.012  1.00 22.52      A
ATOM     61  CD2 LEU A  10      -2.466  24.252  16.753  1.00 24.50      A
ATOM     62  C   LEU A  10      -4.411  20.461  15.818  1.00 18.65      A
ATOM     63  O   LEU A  10      -4.793  20.141  16.945  1.00 16.88      A
ATOM     64  N   VAL A  11      -5.253  20.732  14.830  1.00 15.88      A
ATOM     65  CA  VAL A  11      -6.695  20.633  15.013  1.00 18.59      A
ATOM     66  CB  VAL A  11      -7.455  21.193  13.786  1.00 21.42      A
ATOM     67  CG1 VAL A  11      -8.955  20.957  13.935  1.00 21.23      A
ATOM     68  CG2 VAL A  11      -7.185  22.691  13.659  1.00 23.65      A
ATOM     69  C   VAL A  11      -7.097  19.177  15.250  1.00 18.86      A
ATOM     70  O   VAL A  11      -7.914  18.891  16.116  1.00 19.03      A
ATOM     71  N   ASP A  12      -6.520  18.253  14.489  1.00 19.12      A
ATOM     72  CA  ASP A  12      -6.853  16.845  14.679  1.00 17.94      A
ATOM     73  CB  ASP A  12      -6.173  15.977  13.615  1.00 18.04      A
ATOM     74  CG  ASP A  12      -6.792  16.155  12.239  1.00 18.18      A
ATOM     75  OD1 ASP A  12      -7.888  16.747  12.148  1.00 17.41      A
ATOM     76  OD2 ASP A  12      -6.190  15.696  11.250  1.00 17.12      A
ATOM     77  C   ASP A  12      -6.449  16.374  16.074  1.00 17.94      A
ATOM     78  O   ASP A  12      -7.192  15.636  16.715  1.00 17.22      A
ATOM     79  N   ALA A  13      -5.277  16.804  16.538  1.00 17.64      A
ATOM     80  CA  ALA A  13      -4.796  16.430  17.870  1.00 19.37      A
ATOM     81  CB  ALA A  13      -3.371  16.986  18.101  1.00 13.96      A
ATOM     82  C   ALA A  13      -5.751  16.943  18.952  1.00 19.44      A
ATOM     83  O   ALA A  13      -6.028  16.242  19.925  1.00 22.14      A
ATOM     84  N   LEU A  14      -6.245  18.168  18.783  1.00 21.01      A
ATOM     85  CA  LEU A  14      -7.188  18.760  19.735  1.00 22.46      A
ATOM     86  CB  LEU A  14      -7.482  20.216  19.359  1.00 22.01      A
ATOM     87  CG  LEU A  14      -6.434  21.234  19.809  1.00 24.59      A
ATOM     88  CD1 LEU A  14      -6.611  22.540  19.059  1.00 26.66      A
ATOM     89  CD2 LEU A  14      -6.554  21.439  21.309  1.00 24.62      A
ATOM     90  C   LEU A  14      -8.498  17.976  19.777  1.00 21.46      A
ATOM     91  O   LEU A  14      -9.054  17.715  20.848  1.00 22.17      A
ATOM     92  N   GLN A  15      -8.993  17.615  18.600  1.00 21.30      A
ATOM     93  CA  GLN A  15     -10.224  16.845  18.487  1.00 22.52      A
ATOM     94  CB  GLN A  15     -10.577  16.631  17.010  1.00 21.07      A
ATOM     95  CG  GLN A  15     -11.745  15.682  16.800  1.00 27.80      A
ATOM     96  CD  GLN A  15     -13.071  16.277  17.247  1.00 29.64      A
ATOM     97  OE1 GLN A  15     -13.943  15.568  17.747  1.00 35.24      A
ATOM     98  NE2 GLN A  15     -13.231  17.579  17.057  1.00 28.27      A
ATOM     99  C   GLN A  15     -10.040  15.491  19.174  1.00 22.02      A
ATOM    100  O   GLN A  15     -10.938  15.003  19.860  1.00 22.22      A
ATOM    101  N   PHE A  16      -8.866  14.893  18.997  1.00 21.16      A
ATOM    102  CA  PHE A  16      -8.584  13.596  19.606  1.00 24.00      A
ATOM    103  CB  PHE A  16      -7.271  13.019  19.056  1.00 23.82      A
ATOM    104  CG  PHE A  16      -6.952  11.640  19.578  1.00 29.07      A
ATOM    105  CD1 PHE A  16      -7.795  10.566  19.303  1.00 31.09      A
ATOM    106  CD2 PHE A  16      -5.821  11.419  20.357  1.00 31.17      A
ATOM    107  CE1 PHE A  16      -7.518   9.289  19.796  1.00 32.48      A
ATOM    108  CE2 PHE A  16      -5.532  10.143  20.857  1.00 34.53      A
ATOM    109  CZ  PHE A  16      -6.382   9.078  20.576  1.00 32.86      A
ATOM    110  C   PHE A  16      -8.499  13.703  21.133  1.00 24.10      A
ATOM    111  O   PHE A  16      -9.121  12.927  21.854  1.00 23.16      A
ATOM    112  N   VAL A  17      -7.741  14.677  21.623  1.00 22.26      A
ATOM    113  CA  VAL A  17      -7.578  14.856  23.062  1.00 24.25      A
ATOM    114  CB  VAL A  17      -6.457  15.886  23.360  1.00 25.26      A
ATOM    115  CG1 VAL A  17      -6.397  16.202  24.848  1.00 24.79      A
```

Fig. 5 (contd.)

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ATOM    116  CG2 VAL A  17      -5.126  15.336  22.893  1.00 22.56      A
ATOM    117  C   VAL A  17      -8.859  15.289  23.775  1.00 25.86      A
ATOM    118  O   VAL A  17      -9.221  14.726  24.803  1.00 26.11      A
ATOM    119  N   CYS A  18      -9.551  16.275  23.215  1.00 26.25      A
ATOM    120  CA  CYS A  18     -10.772  16.806  23.819  1.00 27.65      A
ATOM    121  C   CYS A  18     -12.067  16.036  23.553  1.00 29.15      A
ATOM    122  O   CYS A  18     -12.993  16.092  24.358  1.00 31.90      A
ATOM    123  CB  CYS A  18     -10.949  18.263  23.392  1.00 22.29      A
ATOM    124  SG  CYS A  18      -9.517  19.306  23.795  1.00 21.36      A
ATOM    125  N   GLY A  19     -12.144  15.337  22.426  1.00 31.46      A
ATOM    126  CA  GLY A  19     -13.340  14.568  22.121  1.00 31.99      A
ATOM    127  C   GLY A  19     -14.626  15.372  22.043  1.00 34.26      A
ATOM    128  O   GLY A  19     -14.649  16.479  21.504  1.00 35.27      A
ATOM    129  N   ASP A  20     -15.700  14.816  22.594  1.00 34.61      A
ATOM    130  CA  ASP A  20     -17.003  15.470  22.575  1.00 37.53      A
ATOM    131  CB  ASP A  20     -18.094  14.486  23.016  1.00 40.28      A
ATOM    132  CG  ASP A  20     -17.768  13.810  24.322  1.00 44.00      A
ATOM    133  OD1 ASP A  20     -17.483  14.523  25.305  1.00 47.54      A
ATOM    134  OD2 ASP A  20     -17.797  12.563  24.369  1.00 50.02      A
ATOM    135  C   ASP A  20     -17.090  16.743  23.416  1.00 35.72      A
ATOM    136  O   ASP A  20     -18.071  17.475  23.329  1.00 37.26      A
ATOM    137  N   ARG A  21     -16.073  17.016  24.225  1.00 35.25      A
ATOM    138  CA  ARG A  21     -16.083  18.228  25.041  1.00 32.99      A
ATOM    139  CB  ARG A  21     -14.920  18.218  26.028  1.00 33.07      A
ATOM    140  CG  ARG A  21     -14.955  17.084  27.027  1.00 36.04      A
ATOM    141  CD  ARG A  21     -13.650  17.027  27.788  1.00 38.67      A
ATOM    142  NE  ARG A  21     -13.361  18.293  28.449  1.00 40.34      A
ATOM    143  CZ  ARG A  21     -12.237  18.547  29.109  1.00 41.42      A
ATOM    144  NH1 ARG A  21     -11.296  17.618  29.194  1.00 39.64      A
ATOM    145  NH2 ARG A  21     -12.057  19.728  29.688  1.00 42.26      A
ATOM    146  C   ARG A  21     -15.962  19.473  24.166  1.00 31.94      A
ATOM    147  O   ARG A  21     -16.540  20.513  24.465  1.00 32.47      A
ATOM    148  N   GLY A  22     -15.214  19.365  23.075  1.00 29.01      A
ATOM    149  CA  GLY A  22     -15.024  20.521  22.221  1.00 26.06      A
ATOM    150  C   GLY A  22     -13.815  21.261  22.757  1.00 24.26      A
ATOM    151  O   GLY A  22     -13.289  20.890  23.802  1.00 22.46      A
ATOM    152  N   PHE A  23     -13.373  22.306  22.066  1.00 22.86      A
ATOM    153  CA  PHE A  23     -12.198  23.045  22.512  1.00 22.17      A
ATOM    154  CB  PHE A  23     -10.933  22.312  22.039  1.00 20.23      A
ATOM    155  CG  PHE A  23     -10.925  22.004  20.560  1.00 21.99      A
ATOM    156  CD1 PHE A  23     -10.457  22.938  19.642  1.00 22.24      A
ATOM    157  CD2 PHE A  23     -11.414  20.787  20.086  1.00 23.68      A
ATOM    158  CE1 PHE A  23     -10.474  22.670  18.270  1.00 25.86      A
ATOM    159  CE2 PHE A  23     -11.439  20.506  18.713  1.00 25.11      A
ATOM    160  CZ  PHE A  23     -10.968  21.449  17.805  1.00 25.09      A
ATOM    161  C   PHE A  23     -12.181  24.489  22.024  1.00 21.88      A
ATOM    162  O   PHE A  23     -12.973  24.877  21.162  1.00 23.17      A
ATOM    163  N   TYR A  24     -11.274  25.279  22.587  1.00 21.43      A
ATOM    164  CA  TYR A  24     -11.134  26.683  22.215  1.00 22.21      A
ATOM    165  CB  TYR A  24     -11.273  27.587  23.439  1.00 22.91      A
ATOM    166  CG  TYR A  24     -12.633  27.540  24.077  1.00 23.87      A
ATOM    167  CD1 TYR A  24     -13.779  27.855  23.345  1.00 23.25      A
ATOM    168  CE1 TYR A  24     -15.036  27.804  23.928  1.00 23.74      A
ATOM    169  CD2 TYR A  24     -12.781  27.175  25.411  1.00 25.69      A
ATOM    170  CE2 TYR A  24     -14.034  27.125  26.003  1.00 25.28      A
ATOM    171  CZ  TYR A  24     -15.153  27.437  25.260  1.00 26.80      A
ATOM    172  OH  TYR A  24     -16.389  27.374  25.853  1.00 30.71      A
ATOM    173  C   TYR A  24      -9.784  26.938  21.576  1.00 23.50      A
```

Fig. 5 (contd.)

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ATOM    174  O   TYR A  24      -8.853  26.141  21.727  1.00 23.43      A
ATOM    175  N   PHE A  25      -9.682  28.064  20.875  1.00 22.72      A
ATOM    176  CA  PHE A  25      -8.448  28.451  20.208  1.00 25.06      A
ATOM    177  CB  PHE A  25      -8.706  28.776  18.731  1.00 24.67      A
ATOM    178  CG  PHE A  25      -8.965  27.582  17.878  1.00 27.79      A
ATOM    179  CD1 PHE A  25     -10.263  27.237  17.517  1.00 27.21      A
ATOM    180  CD2 PHE A  25      -7.909  26.803  17.417  1.00 28.61      A
ATOM    181  CE1 PHE A  25     -10.506  26.131  16.705  1.00 30.80      A
ATOM    182  CE2 PHE A  25      -8.143  25.692  16.604  1.00 30.69      A
ATOM    183  CZ  PHE A  25      -9.445  25.358  16.248  1.00 28.62      A
ATOM    184  C   PHE A  25      -7.732  29.657  20.804  1.00 26.11      A
ATOM    185  O   PHE A  25      -6.514  29.635  20.992  1.00 26.19      A
ATOM    186  N   ASN A  26      -8.482  30.714  21.095  1.00 27.47      A
ATOM    187  CA  ASN A  26      -7.865  31.948  21.574  1.00 29.42      A
ATOM    188  CB  ASN A  26      -8.809  33.129  21.342  1.00 28.22      A
ATOM    189  CG  ASN A  26      -8.057  34.427  21.126  1.00 31.48      A
ATOM    190  OD1 ASN A  26      -7.194  34.509  20.254  1.00 34.77      A
ATOM    191  ND2 ASN A  26      -8.372  35.441  21.914  1.00 31.59      A
ATOM    192  C   ASN A  26      -7.345  31.989  23.000  1.00 29.47      A
ATOM    193  O   ASN A  26      -6.367  32.680  23.280  1.00 29.14      A
ATOM    194  N   LYS A  27      -7.995  31.263  23.901  1.00 29.57      A
ATOM    195  CA  LYS A  27      -7.569  31.244  25.293  1.00 30.66      A
ATOM    196  CB  LYS A  27      -8.362  32.264  26.118  1.00 30.77      A
ATOM    197  CG  LYS A  27      -8.294  33.694  25.627  1.00 34.21      A
ATOM    198  CD  LYS A  27      -6.919  34.297  25.856  1.00 37.48      A
ATOM    199  CE  LYS A  27      -6.920  35.784  25.530  1.00 40.53      A
ATOM    200  NZ  LYS A  27      -5.582  36.399  25.766  1.00 44.14      A
ATOM    201  C   LYS A  27      -7.821  29.864  25.867  1.00 31.08      A
ATOM    202  O   LYS A  27      -8.707  29.148  25.403  1.00 30.45      A
ATOM    203  N   PRO A  28      -7.032  29.464  26.875  1.00 31.82      A
ATOM    204  CD  PRO A  28      -5.841  30.142  27.421  1.00 32.67      A
ATOM    205  CA  PRO A  28      -7.215  28.152  27.496  1.00 33.47      A
ATOM    206  CB  PRO A  28      -5.895  27.932  28.225  1.00 34.86      A
ATOM    207  CG  PRO A  28      -5.534  29.319  28.659  1.00 34.31      A
ATOM    208  C   PRO A  28      -8.391  28.272  28.459  1.00 35.58      A
ATOM    209  O   PRO A  28      -8.724  29.377  28.900  1.00 32.43      A
ATOM    210  N   THR A  29      -9.025  27.150  28.774  1.00 37.76      A
ATOM    211  CA  THR A  29     -10.154  27.165  29.695  1.00 41.89      A
ATOM    212  CB  THR A  29     -10.940  25.836  29.640  1.00 41.67      A
ATOM    213  OG1 THR A  29     -11.557  25.697  28.353  1.00 43.11      A
ATOM    214  CG2 THR A  29     -12.019  25.805  30.717  1.00 42.37      A
ATOM    215  C   THR A  29      -9.631  27.378  31.110  1.00 45.51      A
ATOM    216  O   THR A  29      -8.550  26.903  31.456  1.00 44.18      A
ATOM    217  N   GLY A  30     -10.390  28.112  31.916  1.00 50.36      A
ATOM    218  CA  GLY A  30      -9.985  28.354  33.288  1.00 56.95      A
ATOM    219  C   GLY A  30      -8.974  29.468  33.469  1.00 61.71      A
ATOM    220  O   GLY A  30      -9.261  30.628  33.172  1.00 63.19      A
ATOM    221  N   TYR A  31      -7.787  29.107  33.955  1.00 65.17      A
ATOM    222  CA  TYR A  31      -6.715  30.067  34.209  1.00 68.15      A
ATOM    223  CB  TYR A  31      -6.541  31.020  33.015  1.00 70.95      A
ATOM    224  CG  TYR A  31      -5.399  32.012  33.154  1.00 73.49      A
ATOM    225  CD1 TYR A  31      -5.486  33.094  34.033  1.00 74.34      A
ATOM    226  CE1 TYR A  31      -4.445  34.004  34.167  1.00 76.14      A
ATOM    227  CD2 TYR A  31      -4.229  31.868  32.406  1.00 74.79      A
ATOM    228  CE2 TYR A  31      -3.177  32.778  32.533  1.00 76.14      A
ATOM    229  CZ  TYR A  31      -3.294  33.842  33.416  1.00 76.89      A
ATOM    230  OH  TYR A  31      -2.264  34.743  33.556  1.00 77.25      A
ATOM    231  C   TYR A  31      -7.058  30.842  35.479  1.00 68.59      A
```

Fig. 5 (contd.)

ATOM	232	OT1	TYR	A	31	-7.492	32.010	35.373	1.00	69.00	A
ATOM	233	OXT	TYR	A	31	-6.913	30.253	36.573	1.00	68.68	A
ATOM	575	CB	THR	C	41	-0.918	33.149	24.393	1.00	42.36	C
ATOM	576	OG1	THR	C	41	-0.923	33.851	23.141	1.00	46.28	C
ATOM	577	CG2	THR	C	41	-0.325	31.766	24.185	1.00	44.57	C
ATOM	578	C	THR	C	41	-3.105	31.956	24.138	1.00	36.92	C
ATOM	579	O	THR	C	41	-3.705	31.060	24.730	1.00	36.68	C
ATOM	580	N	THR	C	41	-2.348	32.688	26.380	1.00	41.52	C
ATOM	581	CA	THR	C	41	-2.354	33.032	24.928	1.00	40.17	C
ATOM	582	N	GLY	C	42	-3.064	32.049	22.809	1.00	33.32	C
ATOM	583	CA	GLY	C	42	-3.763	31.092	21.963	1.00	30.89	C
ATOM	584	C	GLY	C	42	-2.997	29.810	21.687	1.00	29.22	C
ATOM	585	O	GLY	C	42	-1.768	29.789	21.730	1.00	27.67	C
ATOM	586	N	ILE	C	43	-3.726	28.738	21.383	1.00	27.59	C
ATOM	587	CA	ILE	C	43	-3.106	27.441	21.115	1.00	26.54	C
ATOM	588	CB	ILE	C	43	-4.184	26.327	20.976	1.00	26.50	C
ATOM	589	CG2	ILE	C	43	-5.039	26.562	19.734	1.00	25.25	C
ATOM	590	CG1	ILE	C	43	-3.513	24.950	20.910	1.00	28.05	C
ATOM	591	CD	ILE	C	43	-2.830	24.535	22.192	1.00	29.33	C
ATOM	592	C	ILE	C	43	-2.186	27.427	19.878	1.00	26.00	C
ATOM	593	O	ILE	C	43	-1.163	26.742	19.875	1.00	23.28	C
ATOM	594	N	VAL	C	44	-2.544	28.166	18.831	1.00	27.62	C
ATOM	595	CA	VAL	C	44	-1.704	28.221	17.628	1.00	31.20	C
ATOM	596	CB	VAL	C	44	-2.353	29.056	16.502	1.00	32.97	C
ATOM	597	CG1	VAL	C	44	-1.364	29.237	15.353	1.00	33.20	C
ATOM	598	CG2	VAL	C	44	-3.614	28.368	16.001	1.00	33.05	C
ATOM	599	C	VAL	C	44	-0.354	28.848	17.968	1.00	33.88	C
ATOM	600	O	VAL	C	44	0.694	28.350	17.550	1.00	33.44	C
ATOM	601	N	ASP	C	45	-0.383	29.944	18.724	1.00	35.62	C
ATOM	602	CA	ASP	C	45	0.844	30.623	19.132	1.00	36.95	C
ATOM	603	CB	ASP	C	45	0.524	31.847	19.996	1.00	38.58	C
ATOM	604	CG	ASP	C	45	0.486	33.135	19.198	1.00	39.81	C
ATOM	605	OD1	ASP	C	45	-0.356	34.000	19.516	1.00	42.11	C
ATOM	606	OD2	ASP	C	45	1.300	33.291	18.265	1.00	38.39	C
ATOM	607	C	ASP	C	45	1.719	29.668	19.931	1.00	37.42	C
ATOM	608	O	ASP	C	45	2.888	29.467	19.608	1.00	40.61	C
ATOM	609	N	GLU	C	46	1.150	29.082	20.980	1.00	35.14	C
ATOM	610	CA	GLU	C	46	1.903	28.154	21.810	1.00	34.98	C
ATOM	611	CB	GLU	C	46	1.062	27.683	22.999	1.00	35.29	C
ATOM	612	CG	GLU	C	46	0.816	28.736	24.070	1.00	41.36	C
ATOM	613	CD	GLU	C	46	2.103	29.232	24.714	1.00	44.18	C
ATOM	614	OE1	GLU	C	46	2.938	28.388	25.107	1.00	44.06	C
ATOM	615	OE2	GLU	C	46	2.277	30.463	24.833	1.00	44.52	C
ATOM	616	C	GLU	C	46	2.383	26.937	21.027	1.00	33.34	C
ATOM	617	O	GLU	C	46	3.577	26.795	20.765	1.00	33.89	C
ATOM	618	N	CYS	C	47	1.444	26.077	20.637	1.00	29.37	C
ATOM	619	CA	CYS	C	47	1.769	24.844	19.923	1.00	29.96	C
ATOM	620	C	CYS	C	47	2.496	24.937	18.595	1.00	29.21	C
ATOM	621	O	CYS	C	47	3.383	24.132	18.320	1.00	30.38	C
ATOM	622	CB	CYS	C	47	0.504	23.986	19.756	1.00	26.58	C
ATOM	623	SG	CYS	C	47	0.250	22.978	21.239	1.00	26.80	C
ATOM	624	N	CYS	C	48	2.153	25.919	17.777	1.00	28.58	C
ATOM	625	CA	CYS	C	48	2.792	26.029	16.479	1.00	30.29	C
ATOM	626	C	CYS	C	48	4.138	26.760	16.422	1.00	32.48	C
ATOM	627	O	CYS	C	48	5.013	26.394	15.629	1.00	31.64	C
ATOM	628	CB	CYS	C	48	1.825	26.666	15.482	1.00	32.91	C
ATOM	629	SG	CYS	C	48	2.559	26.749	13.832	1.00	41.22	C
ATOM	630	N	PHE	C	49	4.318	27.775	17.261	1.00	32.39	C

Fig. 5 (contd.)

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ATOM    631  CA  PHE C  49       5.559  28.545  17.247  1.00 33.94      C
ATOM    632  CB  PHE C  49       5.232  30.028  17.057  1.00 32.89      C
ATOM    633  CG  PHE C  49       4.520  30.322  15.771  1.00 30.98      C
ATOM    634  CD1 PHE C  49       5.168  30.155  14.552  1.00 30.72      C
ATOM    635  CD2 PHE C  49       3.194  30.737  15.774  1.00 29.66      C
ATOM    636  CE1 PHE C  49       4.504  30.397  13.353  1.00 31.20      C
ATOM    637  CE2 PHE C  49       2.523  30.981  14.584  1.00 29.98      C
ATOM    638  CZ  PHE C  49       3.178  30.810  13.371  1.00 31.19      C
ATOM    639  C   PHE C  49       6.477  28.382  18.452  1.00 34.62      C
ATOM    640  O   PHE C  49       7.656  28.724  18.376  1.00 36.79      C
ATOM    641  N   ARG C  50       5.956  27.859  19.558  1.00 35.31      C
ATOM    642  CA  ARG C  50       6.765  27.687  20.759  1.00 37.16      C
ATOM    643  CB  ARG C  50       6.127  28.456  21.922  1.00 39.61      C
ATOM    644  CG  ARG C  50       6.136  29.980  21.735  1.00 45.48      C
ATOM    645  CD  ARG C  50       5.256  30.688  22.769  1.00 49.13      C
ATOM    646  NE  ARG C  50       5.220  32.139  22.566  1.00 54.47      C
ATOM    647  CZ  ARG C  50       4.361  32.962  23.171  1.00 57.80      C
ATOM    648  NH1 ARG C  50       3.455  32.485  24.018  1.00 58.23      C
ATOM    649  NH2 ARG C  50       4.410  34.271  22.943  1.00 58.43      C
ATOM    650  C   ARG C  50       6.980  26.223  21.149  1.00 37.35      C
ATOM    651  O   ARG C  50       8.011  25.632  20.832  1.00 37.72      C
ATOM    652  N   SER C  51       6.009  25.636  21.836  1.00 36.82      C
ATOM    653  CA  SER C  51       6.123  24.245  22.259  1.00 36.64      C
ATOM    654  CB  SER C  51       6.913  24.160  23.569  1.00 36.90      C
ATOM    655  OG  SER C  51       6.843  22.858  24.116  1.00 42.09      C
ATOM    656  C   SER C  51       4.749  23.614  22.450  1.00 34.49      C
ATOM    657  O   SER C  51       3.830  24.255  22.959  1.00 35.64      C
ATOM    658  N   CYS C  52       4.620  22.353  22.047  1.00 31.83      C
ATOM    659  CA  CYS C  52       3.355  21.636  22.171  1.00 27.84      C
ATOM    660  C   CYS C  52       3.572  20.302  22.862  1.00 27.84      C
ATOM    661  O   CYS C  52       4.584  19.641  22.645  1.00 29.73      C
ATOM    662  CB  CYS C  52       2.765  21.374  20.786  1.00 26.29      C
ATOM    663  SG  CYS C  52       0.950  21.153  20.677  1.00 25.58      C
ATOM    664  N   ASP C  53       2.622  19.926  23.706  1.00 28.33      C
ATOM    665  CA  ASP C  53       2.649  18.649  24.398  1.00 30.31      C
ATOM    666  CB  ASP C  53       3.561  18.679  25.634  1.00 33.16      C
ATOM    667  CG  ASP C  53       3.229  19.806  26.596  1.00 35.20      C
ATOM    668  OD1 ASP C  53       2.036  20.048  26.866  1.00 34.50      C
ATOM    669  OD2 ASP C  53       4.179  20.440  27.100  1.00 39.85      C
ATOM    670  C   ASP C  53       1.216  18.346  24.795  1.00 30.38      C
ATOM    671  O   ASP C  53       0.338  19.207  24.664  1.00 29.48      C
ATOM    672  N   LEU C  54       0.974  17.129  25.266  1.00 29.20      C
ATOM    673  CA  LEU C  54      -0.368  16.725  25.658  1.00 31.11      C
ATOM    674  CB  LEU C  54      -0.374  15.261  26.110  1.00 31.37      C
ATOM    675  CG  LEU C  54      -0.354  14.214  24.991  1.00 34.52      C
ATOM    676  CD1 LEU C  54      -0.302  12.822  25.591  1.00 36.09      C
ATOM    677  CD2 LEU C  54      -1.603  14.361  24.123  1.00 35.95      C
ATOM    678  C   LEU C  54      -1.009  17.589  26.734  1.00 30.74      C
ATOM    679  O   LEU C  54      -2.207  17.870  26.667  1.00 27.74      C
ATOM    680  N   ARG C  55      -0.227  18.012  27.725  1.00 31.56      C
ATOM    681  CA  ARG C  55      -0.782  18.830  28.800  1.00 34.34      C
ATOM    682  CB  ARG C  55       0.251  19.043  29.911  1.00 38.80      C
ATOM    683  CG  ARG C  55      -0.257  19.915  31.051  1.00 47.66      C
ATOM    684  CD  ARG C  55       0.785  20.080  32.153  1.00 55.42      C
ATOM    685  NE  ARG C  55       0.337  21.016  33.183  1.00 61.23      C
ATOM    686  CZ  ARG C  55       1.059  21.368  34.243  1.00 65.09      C
ATOM    687  NH1 ARG C  55       2.273  20.862  34.423  1.00 66.31      C
ATOM    688  NH2 ARG C  55       0.571  22.237  35.120  1.00 66.42      C
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Fig. 5 (contd.)

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ATOM    689  C   ARG C  55      -1.271  20.180  28.282  1.00 32.74      C
ATOM    690  O   ARG C  55      -2.300  20.682  28.726  1.00 32.45      C
ATOM    691  N   ARG C  56      -0.535  20.757  27.336  1.00 31.40      C
ATOM    692  CA  ARG C  56      -0.908  22.041  26.760  1.00 33.43      C
ATOM    693  CB  ARG C  56       0.174  22.509  25.786  1.00 36.65      C
ATOM    694  CG  ARG C  56      -0.072  23.894  25.210  1.00 44.36      C
ATOM    695  CD  ARG C  56       0.139  24.966  26.264  1.00 50.86      C
ATOM    696  NE  ARG C  56       1.538  25.052  26.675  1.00 53.58      C
ATOM    697  CZ  ARG C  56       1.985  25.846  27.641  1.00 56.11      C
ATOM    698  NH1 ARG C  56       1.141  26.627  28.304  1.00 56.49      C
ATOM    699  NH2 ARG C  56       3.277  25.865  27.942  1.00 57.01      C
ATOM    700  C   ARG C  56      -2.250  21.930  26.026  1.00 31.70      C
ATOM    701  O   ARG C  56      -3.121  22.789  26.171  1.00 30.50      C
ATOM    702  N   LEU C  57      -2.408  20.859  25.250  1.00 30.08      C
ATOM    703  CA  LEU C  57      -3.631  20.619  24.485  1.00 28.56      C
ATOM    704  CB  LEU C  57      -3.484  19.337  23.643  1.00 24.71      C
ATOM    705  CG  LEU C  57      -2.498  19.427  22.467  1.00 25.16      C
ATOM    706  CD1 LEU C  57      -2.266  18.052  21.855  1.00 25.45      C
ATOM    707  CD2 LEU C  57      -3.045  20.381  21.417  1.00 25.04      C
ATOM    708  C   LEU C  57      -4.873  20.511  25.366  1.00 28.56      C
ATOM    709  O   LEU C  57      -5.903  21.120  25.077  1.00 26.60      C
ATOM    710  N   GLU C  58      -4.771  19.740  26.444  1.00 29.27      C
ATOM    711  CA  GLU C  58      -5.899  19.546  27.349  1.00 29.45      C
ATOM    712  CB  GLU C  58      -5.539  18.517  28.426  1.00 32.63      C
ATOM    713  CG  GLU C  58      -5.251  17.142  27.858  1.00 41.62      C
ATOM    714  CD  GLU C  58      -5.007  16.101  28.931  1.00 46.45      C
ATOM    715  OE1 GLU C  58      -5.923  15.868  29.748  1.00 47.01      C
ATOM    716  OE2 GLU C  58      -3.901  15.518  28.954  1.00 47.37      C
ATOM    717  C   GLU C  58      -6.379  20.838  28.003  1.00 26.98      C
ATOM    718  O   GLU C  58      -7.548  20.960  28.356  1.00 26.79      C
ATOM    719  N   MET C  59      -5.485  21.805  28.156  1.00 25.43      C
ATOM    720  CA  MET C  59      -5.857  23.079  28.760  1.00 27.07      C
ATOM    721  CB  MET C  59      -4.622  23.965  28.932  1.00 28.76      C
ATOM    722  CG  MET C  59      -3.616  23.475  29.953  1.00 33.93      C
ATOM    723  SD  MET C  59      -2.160  24.548  29.943  1.00 42.31      C
ATOM    724  CE  MET C  59      -2.861  26.092  30.568  1.00 40.03      C
ATOM    725  C   MET C  59      -6.883  23.827  27.907  1.00 26.07      C
ATOM    726  O   MET C  59      -7.644  24.648  28.421  1.00 26.94      C
ATOM    727  N   TYR C  60      -6.904  23.546  26.607  1.00 23.71      C
ATOM    728  CA  TYR C  60      -7.828  24.228  25.707  1.00 24.20      C
ATOM    729  CB  TYR C  60      -7.141  24.536  24.367  1.00 22.53      C
ATOM    730  CG  TYR C  60      -6.054  25.587  24.496  1.00 23.27      C
ATOM    731  CD1 TYR C  60      -4.794  25.257  25.000  1.00 23.79      C
ATOM    732  CE1 TYR C  60      -3.811  26.225  25.170  1.00 25.75      C
ATOM    733  CD2 TYR C  60      -6.301  26.923  24.163  1.00 24.44      C
ATOM    734  CE2 TYR C  60      -5.323  27.903  24.336  1.00 24.69      C
ATOM    735  CZ  TYR C  60      -4.080  27.545  24.839  1.00 26.37      C
ATOM    736  OH  TYR C  60      -3.112  28.503  25.020  1.00 29.55      C
ATOM    737  C   TYR C  60      -9.145  23.507  25.468  1.00 24.59      C
ATOM    738  O   TYR C  60     -10.004  24.015  24.750  1.00 23.53      C
ATOM    739  N   CYS C  61      -9.314  22.327  26.064  1.00 25.69      C
ATOM    740  CA  CYS C  61     -10.568  21.598  25.912  1.00 25.12      C
ATOM    741  C   CYS C  61     -11.642  22.398  26.645  1.00 28.22      C
ATOM    742  O   CYS C  61     -11.346  23.095  27.609  1.00 26.64      C
ATOM    743  CB  CYS C  61     -10.480  20.197  26.538  1.00 23.04      C
ATOM    744  SG  CYS C  61      -9.281  19.035  25.795  1.00 24.30      C
ATOM    745  N   ALA C  62     -12.885  22.305  26.188  1.00 31.44      C
ATOM    746  CA  ALA C  62     -13.976  23.021  26.840  1.00 35.50      C
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Fig. 5 (contd.)

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ATOM    747  CB  ALA C  62     -15.165  23.149  25.889  1.00 34.68      C
ATOM    748  C   ALA C  62     -14.387  22.270  28.114  1.00 39.58      C
ATOM    749  O   ALA C  62     -14.056  21.095  28.287  1.00 38.59      C
ATOM    750  N   PRO C  63     -15.105  22.946  29.029  1.00 43.28      C
ATOM    751  CD  PRO C  63     -15.305  24.404  29.056  1.00 43.94      C
ATOM    752  CA  PRO C  63     -15.559  22.347  30.292  1.00 45.95      C
ATOM    753  CB  PRO C  63     -16.002  23.558  31.116  1.00 46.29      C
ATOM    754  CG  PRO C  63     -15.224  24.694  30.529  1.00 45.50      C
ATOM    755  C   PRO C  63     -16.687  21.319  30.182  1.00 48.67      C
ATOM    756  O   PRO C  63     -17.376  21.235  29.166  1.00 48.01      C
ATOM    757  N   LEU C  64     -16.855  20.568  31.272  1.00 52.88      C
ATOM    758  CA  LEU C  64     -17.870  19.528  31.463  1.00 55.50      C
ATOM    759  CB  LEU C  64     -19.061  19.715  30.522  1.00 56.62      C
ATOM    760  CG  LEU C  64     -19.808  21.040  30.660  1.00 58.22      C
ATOM    761  CD1 LEU C  64     -21.293  20.790  30.430  1.00 59.37      C
ATOM    762  CD2 LEU C  64     -19.580  21.635  32.040  1.00 58.04      C
ATOM    763  C   LEU C  64     -17.324  18.127  31.304  1.00 57.11      C
ATOM    764  OT1 LEU C  64     -16.093  17.996  31.140  1.00 58.74      C
ATOM    765  OXT LEU C  64     -18.143  17.178  31.358  1.00 57.70      C
ATOM    766  OH2 WAT W   1     -13.420  23.772  13.420  1.00 68.77      W
ATOM    767  OH2 WAT W   2       5.123   4.904  12.841  1.00 49.26      W
ATOM    768  OH2 WAT W   3      -9.710   8.110  16.167  1.00 45.79      W
ATOM    769  OH2 WAT W   4       3.964  25.988  25.041  1.00 54.34      W
ATOM    770  OH2 WAT W   5       9.154  10.467  18.403  1.00 53.88      W
ATOM    771  OH2 WAT W   6      -1.831  38.943  35.406  1.00 67.81      W
ATOM    772  OH2 WAT W   7      -3.143   7.399  14.215  1.00 29.35      W
ATOM    773  OH2 WAT W   8     -14.590  17.960  14.582  1.00 46.20      W
ATOM    774  OH2 WAT W   9      -2.241  21.870   7.572  1.00 48.21      W
ATOM    775  OH2 WAT W  10       5.133  17.056  23.108  1.00 30.14      W
ATOM    776  OH2 WAT W  11       2.633  16.411  28.461  1.00 38.79      W
ATOM    777  OH2 WAT W  12      -2.644  31.927  18.931  1.00 37.23      W
ATOM    778  OH2 WAT W  13       3.849  23.009  26.307  1.00 55.69      W
ATOM    779  OH2 WAT W  14      -7.124   5.979  19.437  1.00 36.83      W
ATOM    780  OH2 WAT W  15       9.980   5.519  30.788  1.00 46.86      W
ATOM    781  OH2 WAT W  16     -10.231  17.718  13.498  1.00 26.29      W
ATOM    782  OH2 WAT W  17      -4.408  10.918  24.751  1.00 29.96      W
ATOM    783  OH2 WAT W  18       7.236  20.067  23.369  1.00 42.55      W
ATOM    784  OH2 WAT W  19       3.084  15.212  24.509  1.00 46.38      W
ATOM    785  OH2 WAT W  20      -8.400  32.856  29.980  1.00 60.13      W
ATOM    786  OH2 WAT W  21       2.278   1.311  19.768  1.00 44.76      W
ATOM    787  OH2 WAT W  22      -5.002  30.025  18.420  1.00 36.23      W
ATOM    788  OH2 WAT W  23      -1.894   1.792  15.573  1.00 42.85      W
ATOM    789  OH2 WAT W  24       2.515  20.323  12.519  1.00 42.58      W
ATOM    790  OH2 WAT W  25      -1.706  13.946  10.623  1.00 48.03      W
ATOM    791  OH2 WAT W  26      -7.512   8.258  24.298  1.00 34.80      W
ATOM    792  OH2 WAT W  27     -13.597   3.240  18.060  1.00 44.21      W
ATOM    793  OH2 WAT W  28       8.250  19.107  15.955  1.00 48.54      W
ATOM    794  OH2 WAT W  29       6.930  10.297  13.784  1.00 49.24      W
ATOM    795  OH2 WAT W  30       8.076  20.627  32.202  1.00 66.78      W
ATOM    796  OH2 WAT W  31       0.454  -3.723  20.999  1.00 44.55      W
ATOM    797  OH2 WAT W  32      -8.565   6.277  22.112  1.00 34.63      W
ATOM    798  OH2 WAT W  33       8.280  18.689  11.682  1.00 78.53      W
ATOM    799  OH2 WAT W  34      -8.092   0.771  14.789  1.00 47.22      W
ATOM    800  OH2 WAT W  35      -6.959  25.238  10.263  1.00 54.50      W
ATOM    801  OH2 WAT W  36       6.625   0.437  15.541  1.00 61.08      W
ATOM    802  OH2 WAT W  37     -12.355  24.838  12.355  1.00 34.01      W
ATOM    803  OH2 WAT W  38       8.509   2.752  16.695  1.00 59.59      W
ATOM    804  OH2 WAT W  39       4.162  17.600  11.512  1.00 48.15      W
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Fig. 5 (contd.)

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ATOM    805  OH2 WAT W  40       1.920  -3.265  24.783  1.00 57.34      W
ATOM    806  OH2 WAT W  41      -0.597  19.777   9.560  1.00 40.19      W
ATOM    807  OH2 WAT W  42      -3.808  24.960   7.754  1.00 67.71      W
ATOM    808  OH2 WAT W  43      -7.378  11.115  23.652  1.00 41.42      W
ATOM    809  OH2 WAT W  44      -4.078  -0.471  16.534  1.00 76.11      W
ATOM    810  OH2 WAT W  45      -6.606  27.261  12.681  1.00 48.25      W
ATOM    811  OH2 WAT W  46       6.971  19.806  27.054  1.00 62.02      W
ATOM    812  OH2 WAT W  47      -5.667  31.612  15.738  1.00 53.82      W
END
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Fig. 6

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ATOM    234  CB  SER B  39     -15.365  -2.991  19.079  1.00 59.00      B
ATOM    235  OG  SER B  39     -14.721  -3.902  19.953  1.00 61.35      B
ATOM    236  C   SER B  39     -13.310  -1.576  18.903  1.00 56.28      B
ATOM    237  O   SER B  39     -12.244  -1.252  18.377  1.00 56.57      B
ATOM    238  N   SER B  39     -13.720  -3.401  17.275  1.00 58.47      B
ATOM    239  CA  SER B  39     -14.362  -2.351  18.115  1.00 57.58      B
ATOM    240  N   ALA B  40     -13.611  -1.286  20.164  1.00 53.32      B
ATOM    241  CA  ALA B  40     -12.690  -0.544  21.012  1.00 50.57      B
ATOM    242  CB  ALA B  40     -13.348  -0.230  22.346  1.00 50.99      B
ATOM    243  C   ALA B  40     -11.396  -1.314  21.239  1.00 49.80      B
ATOM    244  O   ALA B  40     -11.406  -2.534  21.407  1.00 50.30      B
ATOM    245  N   LEU B  41     -10.281  -0.591  21.237  1.00 45.57      B
ATOM    246  CA  LEU B  41      -8.971  -1.193  21.454  1.00 43.28      B
ATOM    247  CB  LEU B  41      -7.877  -0.227  20.990  1.00 40.27      B
ATOM    248  CG  LEU B  41      -7.494  -0.187  19.501  1.00 40.88      B
ATOM    249  CD1 LEU B  41      -8.630  -0.665  18.610  1.00 37.72      B
ATOM    250  CD2 LEU B  41      -7.069   1.232  19.143  1.00 37.68      B
ATOM    251  C   LEU B  41      -8.783  -1.539  22.932  1.00 42.24      B
ATOM    252  O   LEU B  41      -9.236  -0.808  23.814  1.00 41.16      B
ATOM    253  N   ALA B  42      -8.113  -2.655  23.200  1.00 41.11      B
ATOM    254  CA  ALA B  42      -7.891  -3.093  24.576  1.00 40.48      B
ATOM    255  CB  ALA B  42      -7.864  -4.618  24.639  1.00 39.75      B
ATOM    256  C   ALA B  42      -6.610  -2.524  25.169  1.00 39.24      B
ATOM    257  O   ALA B  42      -5.830  -1.868  24.477  1.00 38.76      B
ATOM    258  N   GLU B  43      -6.403  -2.771  26.460  1.00 39.27      B
ATOM    259  CA  GLU B  43      -5.213  -2.288  27.150  1.00 38.65      B
ATOM    260  CB  GLU B  43      -5.316  -2.573  28.655  1.00 41.85      B
ATOM    261  CG  GLU B  43      -4.159  -1.998  29.472  1.00 45.40      B
ATOM    262  CD  GLU B  43      -4.231  -2.364  30.948  1.00 47.82      B
ATOM    263  OE1 GLU B  43      -5.227  -2.012  31.614  1.00 50.74      B
ATOM    264  OE2 GLU B  43      -3.283  -3.006  31.444  1.00 49.75      B
ATOM    265  C   GLU B  43      -3.965  -2.962  26.586  1.00 37.56      B
ATOM    266  O   GLU B  43      -3.904  -4.188  26.486  1.00 37.11      B
ATOM    267  N   GLY B  44      -2.974  -2.156  26.218  1.00 35.48      B
ATOM    268  CA  GLY B  44      -1.740  -2.691  25.670  1.00 33.93      B
ATOM    269  C   GLY B  44      -1.711  -2.703  24.152  1.00 32.93      B
ATOM    270  O   GLY B  44      -0.666  -2.932  23.542  1.00 32.61      B
ATOM    271  N   GLN B  45      -2.859  -2.446  23.535  1.00 31.74      B
ATOM    272  CA  GLN B  45      -2.949  -2.447  22.081  1.00 30.82      B
ATOM    273  CB  GLN B  45      -4.373  -2.818  21.653  1.00 31.47      B
ATOM    274  CG  GLN B  45      -4.530  -3.118  20.164  1.00 31.16      B
ATOM    275  CD  GLN B  45      -5.929  -3.615  19.810  1.00 30.41      B
ATOM    276  OE1 GLN B  45      -6.200  -3.967  18.664  1.00 30.09      B
ATOM    277  NE2 GLN B  45      -6.816  -3.650  20.797  1.00 29.72      B
ATOM    278  C   GLN B  45      -2.556  -1.097  21.481  1.00 30.79      B
ATOM    279  O   GLN B  45      -2.806  -0.045  22.067  1.00 31.74      B
ATOM    280  N   SER B  46      -1.930  -1.127  20.310  1.00 29.55      B
ATOM    281  CA  SER B  46      -1.529   0.107  19.647  1.00 29.84      B
ATOM    282  CB  SER B  46      -0.772  -0.208  18.358  1.00 28.36      B
ATOM    283  OG  SER B  46       0.549  -0.617  18.664  1.00 34.06      B
ATOM    284  C   SER B  46      -2.750   0.975  19.347  1.00 27.63      B
ATOM    285  O   SER B  46      -3.851   0.467  19.134  1.00 26.91      B
ATOM    286  N   CYS B  47      -2.543   2.287  19.332  1.00 27.18      B
ATOM    287  CA  CYS B  47      -3.619   3.233  19.083  1.00 25.85      B
ATOM    288  C   CYS B  47      -3.033   4.544  18.572  1.00 26.46      B
ATOM    289  O   CYS B  47      -1.831   4.790  18.685  1.00 28.40      B
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Fig. 6 (contd.)

ATOM	290	CB	CYS	B	47	-4.388	3.499	20.389	1.00	25.30	B
ATOM	291	SG	CYS	B	47	-3.300	4.101	21.724	1.00	28.90	B
ATOM	292	N	GLY	B	48	-3.895	5.389	18.018	1.00	25.42	B
ATOM	293	CA	GLY	B	48	-3.441	6.670	17.518	1.00	24.68	B
ATOM	294	C	GLY	B	48	-4.594	7.619	17.293	1.00	24.57	B
ATOM	295	O	GLY	B	48	-5.754	7.265	17.497	1.00	24.39	B
ATOM	296	N	VAL	B	49	-4.276	8.832	16.866	1.00	25.01	B
ATOM	297	CA	VAL	B	49	-5.297	9.833	16.599	1.00	24.08	B
ATOM	298	CB	VAL	B	49	-4.652	11.139	16.072	1.00	23.73	B
ATOM	299	CG1	VAL	B	49	-5.724	12.123	15.636	1.00	23.71	B
ATOM	300	CG2	VAL	B	49	-3.784	11.748	17.150	1.00	21.54	B
ATOM	301	C	VAL	B	49	-6.320	9.349	15.576	1.00	25.55	B
ATOM	302	O	VAL	B	49	-7.507	9.628	15.701	1.00	27.63	B
ATOM	303	N	TYR	B	50	-5.859	8.603	14.577	1.00	27.40	B
ATOM	304	CA	TYR	B	50	-6.735	8.131	13.510	1.00	28.08	B
ATOM	305	CB	TYR	B	50	-6.067	8.426	12.168	1.00	26.58	B
ATOM	306	CG	TYR	B	50	-5.626	9.868	12.032	1.00	26.76	B
ATOM	307	CD1	TYR	B	50	-6.561	10.888	11.835	1.00	24.56	B
ATOM	308	CE1	TYR	B	50	-6.164	12.218	11.736	1.00	23.17	B
ATOM	309	CD2	TYR	B	50	-4.274	10.218	12.127	1.00	23.57	B
ATOM	310	CE2	TYR	B	50	-3.866	11.546	12.030	1.00	24.76	B
ATOM	311	CZ	TYR	B	50	-4.817	12.541	11.834	1.00	19.86	B
ATOM	312	OH	TYR	B	50	-4.420	13.854	11.736	1.00	20.16	B
ATOM	313	C	TYR	B	50	-7.173	6.663	13.549	1.00	29.31	B
ATOM	314	O	TYR	B	50	-7.685	6.148	12.556	1.00	31.59	B
ATOM	315	N	THR	B	51	-6.981	5.991	14.679	1.00	29.31	B
ATOM	316	CA	THR	B	51	-7.384	4.591	14.800	1.00	28.76	B
ATOM	317	CB	THR	B	51	-6.333	3.742	15.566	1.00	27.30	B
ATOM	318	OG1	THR	B	51	-6.285	4.165	16.935	1.00	27.10	B
ATOM	319	CG2	THR	B	51	-4.944	3.896	14.944	1.00	25.06	B
ATOM	320	C	THR	B	51	-8.672	4.545	15.600	1.00	30.25	B
ATOM	321	O	THR	B	51	-9.198	5.584	15.997	1.00	29.66	B
ATOM	322	N	GLU	B	52	-9.187	3.343	15.832	1.00	32.77	B
ATOM	323	CA	GLU	B	52	-10.393	3.198	16.634	1.00	35.03	B
ATOM	324	CB	GLU	B	52	-10.827	1.730	16.707	1.00	38.95	B
ATOM	325	CG	GLU	B	52	-11.317	1.146	15.395	1.00	42.98	B
ATOM	326	CD	GLU	B	52	-12.554	1.852	14.872	1.00	48.93	B
ATOM	327	OE1	GLU	B	52	-13.516	2.021	15.653	1.00	49.81	B
ATOM	328	OE2	GLU	B	52	-12.566	2.233	13.680	1.00	49.71	B
ATOM	329	C	GLU	B	52	-9.995	3.682	18.021	1.00	34.86	B
ATOM	330	O	GLU	B	52	-8.806	3.718	18.356	1.00	32.99	B
ATOM	331	N	ARG	B	53	-10.977	4.050	18.831	1.00	36.33	B
ATOM	332	CA	ARG	B	53	-10.691	4.528	20.174	1.00	38.61	B
ATOM	333	CB	ARG	B	53	-11.853	5.389	20.675	1.00	43.21	B
ATOM	334	CG	ARG	B	53	-11.988	6.685	19.897	1.00	49.72	B
ATOM	335	CD	ARG	B	53	-13.194	7.500	20.324	1.00	56.57	B
ATOM	336	NE	ARG	B	53	-13.145	7.890	21.729	1.00	61.53	B
ATOM	337	CZ	ARG	B	53	-13.987	8.753	22.288	1.00	63.65	B
ATOM	338	NH1	ARG	B	53	-14.939	9.316	21.556	1.00	64.66	B
ATOM	339	NH2	ARG	B	53	-13.878	9.054	23.575	1.00	65.21	B
ATOM	340	C	ARG	B	53	-10.412	3.397	21.153	1.00	37.36	B
ATOM	341	O	ARG	B	53	-10.854	2.263	20.953	1.00	37.28	B
ATOM	342	N	CYS	B	54	-9.655	3.711	22.201	1.00	35.80	B
ATOM	343	CA	CYS	B	54	-9.324	2.733	23.232	1.00	37.19	B
ATOM	344	C	CYS	B	54	-10.600	2.430	24.001	1.00	39.75	B
ATOM	345	O	CYS	B	54	-11.555	3.205	23.949	1.00	39.46	B
ATOM	346	CB	CYS	B	54	-8.283	3.289	24.210	1.00	32.76	B
ATOM	347	SG	CYS	B	54	-6.595	3.539	23.563	1.00	31.75	B

Fig. 6 (contd.)

ATOM	348	N	ALA	B	55	-10.612	1.311	24.722	1.00	39.90	B
ATOM	349	CA	ALA	B	55	-11.782	0.929	25.496	1.00	42.01	B
ATOM	350	CB	ALA	B	55	-11.620	-0.493	26.035	1.00	40.89	B
ATOM	351	C	ALA	B	55	-11.980	1.909	26.643	1.00	43.33	B
ATOM	352	O	ALA	B	55	-11.035	2.562	27.090	1.00	44.41	B
ATOM	353	N	GLN	B	56	-13.217	2.005	27.114	1.00	46.27	B
ATOM	354	CA	GLN	B	56	-13.569	2.900	28.209	1.00	46.98	B
ATOM	355	CB	GLN	B	56	-15.020	2.661	28.631	1.00	49.45	B
ATOM	356	CG	GLN	B	56	-16.030	2.703	27.487	1.00	52.92	B
ATOM	357	CD	GLN	B	56	-16.073	1.414	26.671	1.00	55.64	B
ATOM	358	OE1	GLN	B	56	-15.076	0.999	26.077	1.00	54.71	B
ATOM	359	NE2	GLN	B	56	-17.240	0.777	26.641	1.00	55.29	B
ATOM	360	C	GLN	B	56	-12.649	2.685	29.405	1.00	46.86	B
ATOM	361	O	GLN	B	56	-12.315	1.549	29.746	1.00	46.71	B
ATOM	362	N	GLY	B	57	-12.242	3.779	30.040	1.00	46.46	B
ATOM	363	CA	GLY	B	57	-11.366	3.679	31.193	1.00	45.89	B
ATOM	364	C	GLY	B	57	-9.903	3.715	30.799	1.00	46.63	B
ATOM	365	O	GLY	B	57	-9.017	3.747	31.653	1.00	47.54	B
ATOM	366	N	LEU	B	58	-9.650	3.707	29.496	1.00	45.76	B
ATOM	367	CA	LEU	B	58	-8.290	3.745	28.974	1.00	44.32	B
ATOM	368	CB	LEU	B	58	-7.982	2.450	28.212	1.00	45.02	B
ATOM	369	CG	LEU	B	58	-8.134	1.093	28.908	1.00	45.27	B
ATOM	370	CD1	LEU	B	58	-8.043	-0.017	27.872	1.00	44.01	B
ATOM	371	CD2	LEU	B	58	-7.060	0.928	29.970	1.00	44.12	B
ATOM	372	C	LEU	B	58	-8.153	4.923	28.009	1.00	43.40	B
ATOM	373	O	LEU	B	58	-9.143	5.390	27.441	1.00	42.14	B
ATOM	374	N	ARG	B	59	-6.929	5.407	27.836	1.00	40.99	B
ATOM	375	CA	ARG	B	59	-6.674	6.487	26.899	1.00	41.20	B
ATOM	376	CB	ARG	B	59	-6.593	7.853	27.604	1.00	42.80	B
ATOM	377	CG	ARG	B	59	-5.456	8.032	28.582	1.00	48.53	B
ATOM	378	CD	ARG	B	59	-5.366	9.484	29.058	1.00	50.94	B
ATOM	379	NE	ARG	B	59	-5.012	10.404	27.977	1.00	54.00	B
ATOM	380	CZ	ARG	B	59	-4.944	11.728	28.108	1.00	55.67	B
ATOM	381	NH1	ARG	B	59	-5.208	12.300	29.276	1.00	55.62	B
ATOM	382	NH2	ARG	B	59	-4.607	12.485	27.070	1.00	54.65	B
ATOM	383	C	ARG	B	59	-5.382	6.183	26.149	1.00	38.98	B
ATOM	384	O	ARG	B	59	-4.509	5.465	26.648	1.00	37.23	B
ATOM	385	N	CYS	B	60	-5.277	6.724	24.941	1.00	34.30	B
ATOM	386	CA	CYS	B	60	-4.121	6.504	24.097	1.00	34.00	B
ATOM	387	C	CYS	B	60	-2.931	7.400	24.451	1.00	34.57	B
ATOM	388	O	CYS	B	60	-2.995	8.617	24.305	1.00	34.82	B
ATOM	389	CB	CYS	B	60	-4.527	6.698	22.630	1.00	31.12	B
ATOM	390	SG	CYS	B	60	-3.259	6.122	21.472	1.00	28.31	B
ATOM	391	N	LEU	B	61	-1.846	6.785	24.919	1.00	35.17	B
ATOM	392	CA	LEU	B	61	-0.637	7.516	25.295	1.00	37.27	B
ATOM	393	CB	LEU	B	61	-0.345	7.379	26.794	1.00	36.88	B
ATOM	394	CG	LEU	B	61	-1.281	8.031	27.804	1.00	39.20	B
ATOM	395	CD1	LEU	B	61	-1.388	9.534	27.548	1.00	37.47	B
ATOM	396	CD2	LEU	B	61	-2.625	7.367	27.702	1.00	40.69	B
ATOM	397	C	LEU	B	61	0.571	7.005	24.539	1.00	37.81	B
ATOM	398	O	LEU	B	61	0.640	5.830	24.187	1.00	37.72	B
ATOM	399	N	PRO	B	62	1.553	7.885	24.293	1.00	40.67	B
ATOM	400	CD	PRO	B	62	1.530	9.335	24.565	1.00	42.50	B
ATOM	401	CA	PRO	B	62	2.771	7.512	23.573	1.00	43.32	B
ATOM	402	CB	PRO	B	62	3.245	8.843	23.011	1.00	42.71	B
ATOM	403	CG	PRO	B	62	2.918	9.781	24.133	1.00	41.70	B
ATOM	404	C	PRO	B	62	3.806	6.884	24.502	1.00	45.58	B
ATOM	405	O	PRO	B	62	3.670	6.939	25.723	1.00	43.82	B

Fig. 6 (contd.)

ATOM	406	N	ARG	B	63	4.834	6.283	23.913	1.00	48.66	B
ATOM	407	CA	ARG	B	63	5.910	5.663	24.678	1.00	52.24	B
ATOM	408	CB	ARG	B	63	6.805	4.830	23.757	1.00	54.66	B
ATOM	409	CG	ARG	B	63	6.073	4.003	22.705	1.00	57.98	B
ATOM	410	CD	ARG	B	63	5.569	2.675	23.239	1.00	60.28	B
ATOM	411	NE	ARG	B	63	5.043	1.841	22.159	1.00	63.63	B
ATOM	412	CZ	ARG	B	63	4.710	0.561	22.294	1.00	64.74	B
ATOM	413	NH1	ARG	B	63	4.847	-0.043	23.467	1.00	64.62	B
ATOM	414	NH2	ARG	B	63	4.239	-0.116	21.254	1.00	64.94	B
ATOM	415	C	ARG	B	63	6.731	6.811	25.261	1.00	53.02	B
ATOM	416	O	ARG	B	63	7.001	7.790	24.565	1.00	52.38	B
ATOM	417	N	GLN	B	64	7.132	6.694	26.524	1.00	54.59	B
ATOM	418	CA	GLN	B	64	7.918	7.746	27.161	1.00	56.19	B
ATOM	419	CB	GLN	B	64	8.112	7.454	28.654	1.00	58.46	B
ATOM	420	CG	GLN	B	64	6.844	7.599	29.483	1.00	62.16	B
ATOM	421	CD	GLN	B	64	7.117	7.649	30.977	1.00	64.31	B
ATOM	422	OE1	GLN	B	64	6.189	7.677	31.787	1.00	65.56	B
ATOM	423	NE2	GLN	B	64	8.393	7.669	31.349	1.00	65.48	B
ATOM	424	C	GLN	B	64	9.278	7.975	26.508	1.00	54.95	B
ATOM	425	O	GLN	B	64	9.976	8.924	26.852	1.00	55.19	B
ATOM	426	N	ASP	B	65	9.653	7.117	25.566	1.00	54.23	B
ATOM	427	CA	ASP	B	65	10.936	7.273	24.888	1.00	54.67	B
ATOM	428	CB	ASP	B	65	11.622	5.914	24.714	1.00	56.29	B
ATOM	429	CG	ASP	B	65	10.868	4.995	23.776	1.00	57.63	B
ATOM	430	OD1	ASP	B	65	9.738	4.586	24.119	1.00	57.64	B
ATOM	431	OD2	ASP	B	65	11.407	4.684	22.693	1.00	58.07	B
ATOM	432	C	ASP	B	65	10.795	7.952	23.524	1.00	54.03	B
ATOM	433	O	ASP	B	65	11.772	8.089	22.786	1.00	54.88	B
ATOM	434	N	GLU	B	66	9.581	8.376	23.188	1.00	52.05	B
ATOM	435	CA	GLU	B	66	9.346	9.044	21.912	1.00	50.06	B
ATOM	436	CB	GLU	B	66	7.841	9.145	21.629	1.00	47.94	B
ATOM	437	CG	GLU	B	66	7.164	7.825	21.284	1.00	45.31	B
ATOM	438	CD	GLU	B	66	7.504	7.334	19.885	1.00	44.11	B
ATOM	439	OE1	GLU	B	66	8.316	7.987	19.197	1.00	44.31	B
ATOM	440	OE2	GLU	B	66	6.958	6.291	19.468	1.00	43.74	B
ATOM	441	C	GLU	B	66	9.952	10.443	21.938	1.00	49.71	B
ATOM	442	O	GLU	B	66	9.778	11.183	22.905	1.00	49.58	B
ATOM	443	N	GLU	B	67	10.668	10.799	20.876	1.00	49.99	B
ATOM	444	CA	GLU	B	67	11.275	12.123	20.775	1.00	50.53	B
ATOM	445	CB	GLU	B	67	12.245	12.170	19.591	1.00	53.83	B
ATOM	446	CG	GLU	B	67	13.195	10.979	19.489	1.00	59.06	B
ATOM	447	CD	GLU	B	67	14.234	10.942	20.595	1.00	62.91	B
ATOM	448	OE1	GLU	B	67	13.845	10.864	21.780	1.00	64.79	B
ATOM	449	OE2	GLU	B	67	15.443	10.987	20.276	1.00	64.81	B
ATOM	450	C	GLU	B	67	10.139	13.125	20.547	1.00	48.30	B
ATOM	451	O	GLU	B	67	10.140	14.229	21.092	1.00	47.77	B
ATOM	452	N	LYS	B	68	9.170	12.715	19.733	1.00	44.89	B
ATOM	453	CA	LYS	B	68	8.010	13.537	19.404	1.00	41.91	B
ATOM	454	CB	LYS	B	68	7.980	13.821	17.903	1.00	44.05	B
ATOM	455	CG	LYS	B	68	9.325	14.192	17.299	1.00	48.60	B
ATOM	456	CD	LYS	B	68	9.857	15.501	17.840	1.00	51.03	B
ATOM	457	CE	LYS	B	68	11.163	15.858	17.156	1.00	54.19	B
ATOM	458	NZ	LYS	B	68	11.008	15.877	15.673	1.00	54.98	B
ATOM	459	C	LYS	B	68	6.757	12.753	19.786	1.00	36.81	B
ATOM	460	O	LYS	B	68	6.151	12.096	18.944	1.00	36.07	B
ATOM	461	N	PRO	B	69	6.352	12.814	21.064	1.00	33.10	B
ATOM	462	CD	PRO	B	69	6.900	13.675	22.126	1.00	31.55	B
ATOM	463	CA	PRO	B	69	5.166	12.094	21.540	1.00	30.73	B

Fig. 6 (contd.)

ATOM	464	CB	PRO	B	69	5.032	12.557	22.993	1.00	29.25	B
ATOM	465	CG	PRO	B	69	6.414	12.979	23.366	1.00	33.62	B
ATOM	466	C	PRO	B	69	3.895	12.383	20.738	1.00	28.46	B
ATOM	467	O	PRO	B	69	3.145	11.466	20.407	1.00	26.39	B
ATOM	468	N	LEU	B	70	3.647	13.657	20.445	1.00	26.72	B
ATOM	469	CA	LEU	B	70	2.447	14.033	19.700	1.00	27.00	B
ATOM	470	CB	LEU	B	70	2.311	15.559	19.632	1.00	25.43	B
ATOM	471	CG	LEU	B	70	2.028	16.231	20.981	1.00	25.63	B
ATOM	472	CD1	LEU	B	70	1.938	17.740	20.801	1.00	26.23	B
ATOM	473	CD2	LEU	B	70	0.727	15.684	21.565	1.00	23.13	B
ATOM	474	C	LEU	B	70	2.485	13.438	18.306	1.00	26.72	B
ATOM	475	O	LEU	B	70	1.488	12.881	17.829	1.00	25.47	B
ATOM	476	N	HIS	B	71	3.638	13.545	17.653	1.00	26.27	B
ATOM	477	CA	HIS	B	71	3.787	12.978	16.322	1.00	26.43	B
ATOM	478	CB	HIS	B	71	5.129	13.384	15.702	1.00	26.14	B
ATOM	479	CG	HIS	B	71	5.135	14.777	15.153	1.00	28.44	B
ATOM	480	CD2	HIS	B	71	4.945	15.236	13.893	1.00	29.08	B
ATOM	481	ND1	HIS	B	71	5.291	15.893	15.947	1.00	28.51	B
ATOM	482	CE1	HIS	B	71	5.196	16.979	15.201	1.00	28.30	B
ATOM	483	NE2	HIS	B	71	4.985	16.607	13.951	1.00	30.59	B
ATOM	484	C	HIS	B	71	3.654	11.457	16.381	1.00	25.51	B
ATOM	485	O	HIS	B	71	3.177	10.841	15.434	1.00	26.07	B
ATOM	486	N	ALA	B	72	4.048	10.853	17.499	1.00	25.45	B
ATOM	487	CA	ALA	B	72	3.939	9.400	17.643	1.00	26.65	B
ATOM	488	CB	ALA	B	72	4.620	8.936	18.927	1.00	26.26	B
ATOM	489	C	ALA	B	72	2.467	8.996	17.660	1.00	27.13	B
ATOM	490	O	ALA	B	72	2.083	7.976	17.086	1.00	26.66	B
ATOM	491	N	LEU	B	73	1.650	9.805	18.327	1.00	26.56	B
ATOM	492	CA	LEU	B	73	0.219	9.552	18.423	1.00	27.39	B
ATOM	493	CB	LEU	B	73	-0.412	10.502	19.442	1.00	30.38	B
ATOM	494	CG	LEU	B	73	-0.845	9.899	20.780	1.00	35.02	B
ATOM	495	CD1	LEU	B	73	0.235	8.981	21.334	1.00	37.66	B
ATOM	496	CD2	LEU	B	73	-1.146	11.031	21.749	1.00	34.89	B
ATOM	497	C	LEU	B	73	-0.458	9.717	17.069	1.00	24.51	B
ATOM	498	O	LEU	B	73	-1.336	8.939	16.703	1.00	24.68	B
ATOM	499	N	LEU	B	74	-0.044	10.737	16.327	1.00	25.25	B
ATOM	500	CA	LEU	B	74	-0.598	10.988	15.005	1.00	23.89	B
ATOM	501	CB	LEU	B	74	-0.006	12.280	14.423	1.00	22.63	B
ATOM	502	CG	LEU	B	74	-0.503	13.563	15.113	1.00	24.73	B
ATOM	503	CD1	LEU	B	74	0.428	14.725	14.804	1.00	24.97	B
ATOM	504	CD2	LEU	B	74	-1.937	13.864	14.663	1.00	23.07	B
ATOM	505	C	LEU	B	74	-0.287	9.807	14.090	1.00	26.28	B
ATOM	506	O	LEU	B	74	-1.095	9.440	13.235	1.00	23.75	B
ATOM	507	N	HIS	B	75	0.879	9.198	14.290	1.00	26.07	B
ATOM	508	CA	HIS	B	75	1.289	8.071	13.468	1.00	30.78	B
ATOM	509	CB	HIS	B	75	2.789	8.173	13.178	1.00	33.64	B
ATOM	510	CG	HIS	B	75	3.162	9.419	12.438	1.00	38.80	B
ATOM	511	CD2	HIS	B	75	3.870	10.507	12.821	1.00	40.91	B
ATOM	512	ND1	HIS	B	75	2.718	9.683	11.160	1.00	41.10	B
ATOM	513	CE1	HIS	B	75	3.133	10.881	10.789	1.00	41.21	B
ATOM	514	NE2	HIS	B	75	3.834	11.403	11.779	1.00	44.19	B
ATOM	515	C	HIS	B	75	0.935	6.703	14.052	1.00	32.10	B
ATOM	516	O	HIS	B	75	1.539	5.695	13.698	1.00	33.73	B
ATOM	517	N	GLY	B	76	-0.051	6.681	14.943	1.00	32.63	B
ATOM	518	CA	GLY	B	76	-0.508	5.436	15.541	1.00	32.02	B
ATOM	519	C	GLY	B	76	0.471	4.625	16.374	1.00	34.41	B
ATOM	520	O	GLY	B	76	0.343	3.399	16.446	1.00	34.43	B
ATOM	521	N	ARG	B	77	1.434	5.285	17.013	1.00	32.88	B

Fig. 6 (contd.)

```
ATOM    522  CA  ARG B  77       2.410   4.580  17.837  1.00 34.75      B
ATOM    523  CB  ARG B  77       3.828   5.075  17.538  1.00 35.67      B
ATOM    524  CG  ARG B  77       4.297   4.757  16.126  1.00 37.36      B
ATOM    525  CD  ARG B  77       5.807   4.695  16.057  1.00 37.48      B
ATOM    526  NE  ARG B  77       6.435   5.956  16.436  1.00 38.77      B
ATOM    527  CZ  ARG B  77       6.610   6.985  15.613  1.00 38.84      B
ATOM    528  NH1 ARG B  77       6.204   6.909  14.355  1.00 38.80      B
ATOM    529  NH2 ARG B  77       7.202   8.090  16.047  1.00 38.47      B
ATOM    530  C   ARG B  77       2.125   4.701  19.329  1.00 34.86      B
ATOM    531  O   ARG B  77       2.996   4.458  20.161  1.00 36.41      B
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ATOM    534  C   GLY B  78       0.087   3.823  21.546  1.00 31.67      B
ATOM    535  O   GLY B  78       0.032   2.871  20.767  1.00 31.30      B
ATOM    536  N   VAL B  79      -0.239   3.721  22.824  1.00 31.21      B
ATOM    537  CA  VAL B  79      -0.711   2.466  23.393  1.00 33.83      B
ATOM    538  CB  VAL B  79       0.443   1.700  24.101  1.00 36.30      B
ATOM    539  CG1 VAL B  79       1.096   2.589  25.140  1.00 39.47      B
ATOM    540  CG2 VAL B  79      -0.094   0.436  24.760  1.00 38.17      B
ATOM    541  C   VAL B  79      -1.811   2.774  24.403  1.00 32.69      B
ATOM    542  O   VAL B  79      -1.735   3.768  25.125  1.00 34.48      B
ATOM    543  N   CYS B  80      -2.842   1.939  24.432  1.00 32.38      B
ATOM    544  CA  CYS B  80      -3.942   2.129  25.365  1.00 34.85      B
ATOM    545  C   CYS B  80      -3.486   1.739  26.768  1.00 37.39      B
ATOM    546  O   CYS B  80      -3.119   0.591  27.005  1.00 35.79      B
ATOM    547  CB  CYS B  80      -5.144   1.270  24.964  1.00 32.27      B
ATOM    548  SG  CYS B  80      -5.879   1.648  23.342  1.00 34.08      B
ATOM    549  N   LEU B  81      -3.509   2.700  27.689  1.00 42.01      B
ATOM    550  CA  LEU B  81      -3.091   2.463  29.070  1.00 46.11      B
ATOM    551  CB  LEU B  81      -1.729   3.114  29.337  1.00 46.36      B
ATOM    552  CG  LEU B  81      -0.510   2.625  28.548  1.00 47.24      B
ATOM    553  CD1 LEU B  81       0.680   3.510  28.871  1.00 47.79      B
ATOM    554  CD2 LEU B  81      -0.206   1.174  28.885  1.00 45.42      B
ATOM    555  C   LEU B  81      -4.095   2.988  30.093  1.00 49.50      B
ATOM    556  O   LEU B  81      -5.128   3.556  29.741  1.00 48.23      B
ATOM    557  N   ASN B  82      -3.750   2.790  31.363  1.00 54.82      B
ATOM    558  CA  ASN B  82      -4.541   3.195  32.527  1.00 59.40      B
ATOM    559  CB  ASN B  82      -3.602   3.443  33.711  1.00 61.17      B
ATOM    560  CG  ASN B  82      -2.493   4.426  33.378  1.00 62.57      B
ATOM    561  OD1 ASN B  82      -2.714   5.414  32.677  1.00 64.48      B
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ATOM    563  C   ASN B  82      -5.487   4.394  32.410  1.00 61.49      B
ATOM    564  O   ASN B  82      -6.503   4.339  31.719  1.00 61.26      B
ATOM    565  N   GLU B  83      -5.141   5.458  33.134  1.00 64.37      B
ATOM    566  CA  GLU B  83      -5.910   6.700  33.202  1.00 66.99      B
ATOM    567  CB  GLU B  83      -4.964   7.905  33.200  1.00 68.36      B
ATOM    568  CG  GLU B  83      -3.745   7.748  34.100  1.00 70.54      B
ATOM    569  CD  GLU B  83      -4.079   7.137  35.448  1.00 71.94      B
ATOM    570  OE1 GLU B  83      -4.920   7.708  36.172  1.00 73.55      B
ATOM    571  OE2 GLU B  83      -3.496   6.084  35.783  1.00 72.89      B
ATOM    572  C   GLU B  83      -6.939   6.864  32.096  1.00 67.27      B
ATOM    573  OT1 GLU B  83      -8.128   7.052  32.426  1.00 68.26      B
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Fig. 7

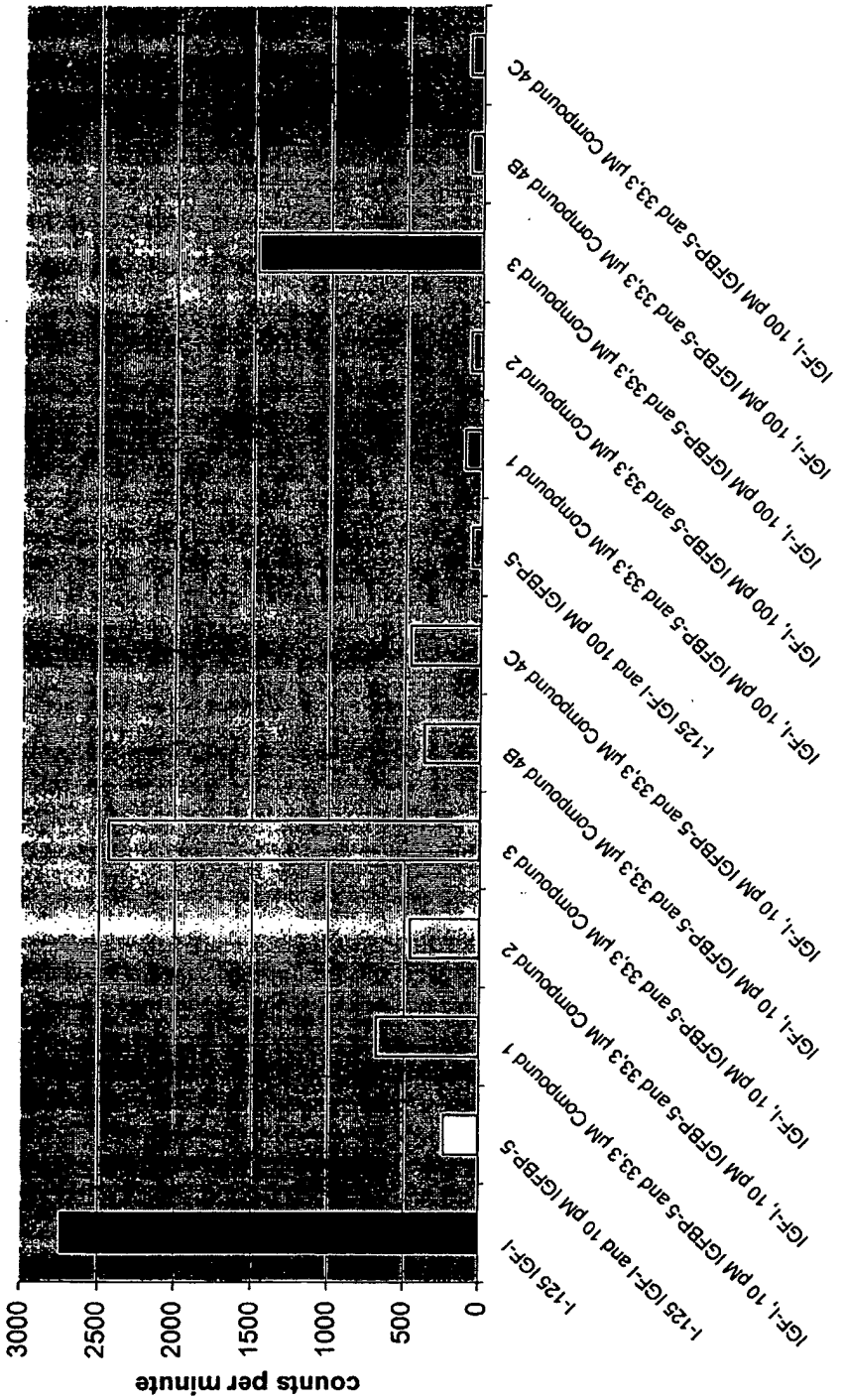
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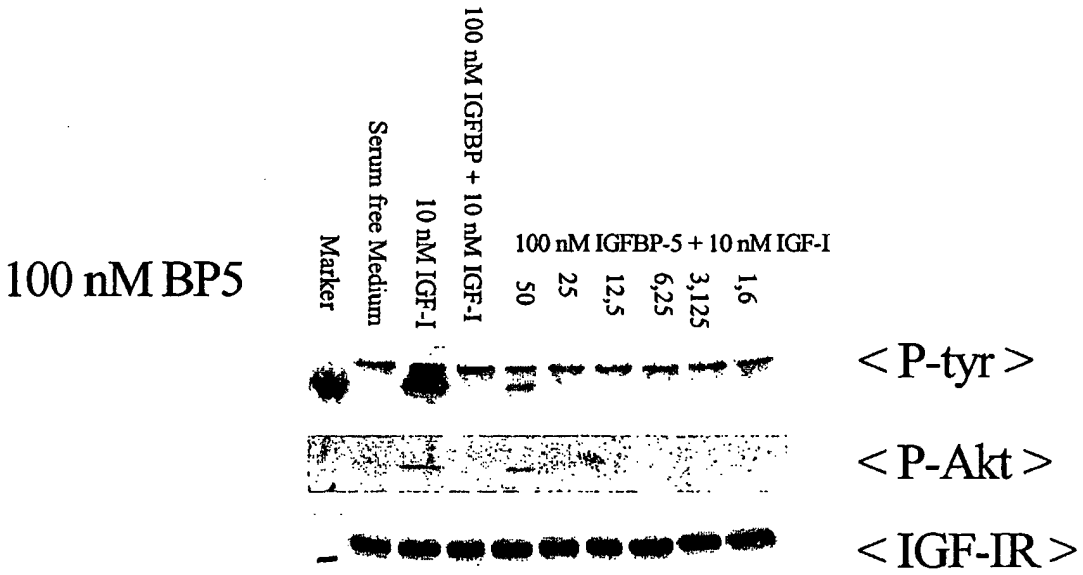
Fig. 8



IGFBP-5 and Compound 3

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